# PHYTOCHEMICAL SCREENING AND EVALUATION OF ANTI-ANAEMIC ACTIVITY OF ENTIRE PLANT OF *Hybanthus enneaspermus* (LINN.) F. MUELL.,

A Dissertation submitted to

**THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY**

**CHENNAI-32**

*In partial fulfilment of the requirements for the award of the degree of*

**MASTER OF PHARMACY IN**

**PHARMACOGNOSY**

*Submitted by*

Reg.No.261420659

*Under the guidance of*

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**MADRAS MEDICAL COLLEGE**

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**APRIL – 2016**

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# CERTIFICATE

This is to certify that the dissertation entitled **"PHYTOCHEMICAL SCREENING AND EVALUATION OF ANTI-ANAEMIC ACTIVITY OF ENTIRE PLANT OF *Hybanthus enneaspermus* (LINN.) F. MUELL.,"** submitted by **Reg.No: 261420659**in partial fulfillment of the requirements for the award of the degree of **MASTER OF PHARMACY IN PHARMACOGNOSY** by The Tamil Nadu Dr.M.G.R. Medical University, Chennai, is a bonafide record of work done by her in the Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai-600003, during the academic year 2015- 2016under the guidance of **Dr.R.RADHA M.Pharm.,Ph.D.,** Department of Pharmacognosy, college of pharmacy, Madras Medical College, Chennai-600003.

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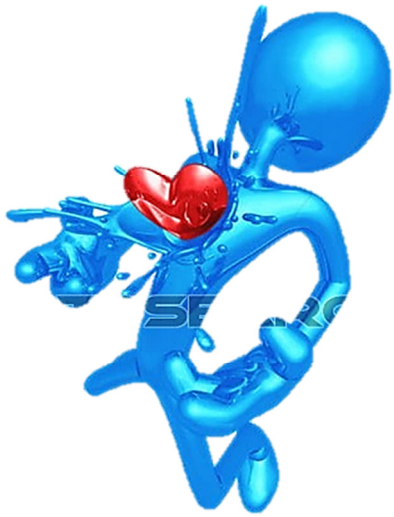


DEDICATED TO MY BELOVED

PARENTS

# ACKNOWLEDGEMENT

I take immense pleasure in extending my gratitude to each and every one who was constantly encouraging me and The Almighty for guiding me with wisdom and support throughout my project work.

I whole heartedly express my high esteem and deep sense of gratitude to respectable **Dr.R.Vimala, M.D.,** DEAN, Madras Medical College, Chennai, for providing me all the facilities and support during the period of academic course work.

In taking great pleasure to thank our beloved Principal **Dr.A.JERADSURESH, M.Pharm.,Ph.D.,M.B.A.,** College of Pharmacy, Madras Medical College, for providing lab and administrative facilities to carry out the project work.

I take this opportunity with profound privilege and great pleasure in expressing my deep sense of gratitude to my respected Guide **Dr.R.RADHA M.Pharm.,Ph.D.,** professor and Head**,** Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai -03, for her gracious guidance, innovative ideas, with constant inspiration, encouragement, suggestions and infinite help throughout my research work. I greatly thank her valuable support and endless consideration of my project work.

I express my deep sense of gratitude to **Dr.N.Jayshree M.Pharm.,Ph.D.,** Professor and Head, Institute of pharmacology, Madras Medical College for her encouragement, love and support for me in completion of my project.

I take great pleasure in acknowledging my sincere thanks to all the staff members **Dr.P.Muthusamy M.Pharm.,Ph.D.,B.L., Dr.R.Vijayabharathi M.Pharm.,Ph.D., Dr.R.Vadivu M.Pharm.,Ph.D.,** of the Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai -03 for their valuable suggestions and moral support.

I extend my gratitude to **Dr.Seenivelan, B.V.sc.,** Special veterinary officer and **Mr.Kandhasamy** and **Mr.Kumar** Assistants of Animal Experiment House, Madras Medical College, Chennai-03 for providing animals to carryout Pharmacological studies.

I acknowledge my sincere thanks to **Dr.Aravind M.D.(s).M.sc.,** Assistant Professor, Department of Medicinal Botany, National Institute of Siddha,Chennai-47, for the authentication of the plant material.

I wish to thank **The Director** Institute of Pharmacology Madras Medical College, Chennai-03 for providing the lab to carryout Pharmacological studies.

I would like to thank **Mrs.V.Geetha** manager-Quality control ATOZ pharmaceuticals Pvt. Ltd., for providing facilities to carryout quantitative studies.

I wish to thank **Department of Microbiology**, Madras medical College in proving lab facilities to carry out blood analysis.

I extend my hearty gratitude to my beloved brother **Dr.M.Samuel** MD(Acu), Ph.D, DHMS, DDT, DHP., for helping me in selecting the Medicinal plant that has initiated my project.

I take my pleasure in thanking my own lovable brother **Mr. S.Karthick BCA.,** for helping me financially for purchasing plant materials.

I express my sincere thanks to my senior **Mr. K.Durai Prabakaran** for his timely help during isolation of active constituent.

I express my hearty thanks to my **Batch mates** for their encouragement and support during project work.

I thank all my juniors for their support and especially **Miss. R.Kopperundevi** for her help during UV analysis.

I express my special thanks to my friend **Miss. M.Kavitha** for sharing suggestions and constantly supporting me throughout the project.

I extend my sincere thanks to my friends of pharmacology department **L.Gowtham, R.Leela, C.M.Maran, S.Mala** and **Saravanan** for helping and encouraging me throughout the project.

I take this opportunity to acknowledge **Mr.UdayaChandran** DTP unit, Co-op stores, MMC, for making my project as a dissertation.

I also extend my thanks to all those who helped me directly or indirectly during this project.

With all my love I extend my hearty gratitude to my **Mother** and **Father** for expressing their love, care, prayer, encouragement and support in all my endeavours throughout life.

# CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# Introduction

# 1. INTRODUCTION

India has rich heritage of its medicinal system with abundant herbs and minerals to treat and cure diseases. Now a days even though the technologies have made it possible to discover new drugs there is always a question of safety and fear of side effects. In this modern era, it is very pathetic that anaemia is still a major killer of India.

Anaemia is the blood disorder characterized by the reduction in RBC, haemoglobin content and packed cell volume which causes symptoms like tachycardia, increase rate and force of respiration, anorexia, nausea, vomiting, disturbance in the menstrual cycle, menorrhagia, oligomenorrhea or amenorrhea, neuromuscular symptoms like headache, lack of concentration, restlessness, drowsiness, dizziness, increased sensitivity to cold and fainting sensation, lack of energy and fatigue.[1]

The following statistics gives an alarming picture about anaemia:

- 56% of Indian women suffer from anaemia[2]
- 4 out of 5 children in the age of 6-35 months suffer from anaemia[3]
- 20% of maternal deaths are due to anaemia and anaemia indirectly contributes to another 40% of maternal deaths and maternal mortality is staggeringly high at 454 per every 100000 live births.[4]
- Anaemia spares none; it affects both adults and children of both sex.

Indian herbs like *Withania somnifera*, *Moringa olifera*, *Neolamrickia cadamba, Phylanthus emblica* etc. which have various medicinal properties can contribute to overcome this pathetic condition.

## ANAEMIA

Anaemia is defined as a normal plasma (14-16g/dl) haemoglobin concentration resulting from a decreased number of circulating red blood cells or an abnormally low total haemoglobin content per unit of blood volume. Anaemia can be caused by chronic blood loss, bone marrow abnormalities, increased haemolysis, infections, malignancy, endocrine deficiencies, renal failure and a number of other disease states. A large number of drugs cause toxic effects on blood cells, haemoglobin production or erythropoietic organs, which in turn, may cause anaemia. In addition, nutritional anaemias are caused by dietary deficiencies of substance such as iron, folic acid, and vitamin $B_{12}$ (cynocobalamine) that are necessary for normal erythropoiesis. [5]

### WHO definition for anaemia

Anaemia is a condition in which the number of red blood cells or their oxygen carrying capacity is insufficient to meet physiologic needs, which vary by age, sex, altitude, smoking and pregnancy status.

In its severe form, it is associated with fatigue, weakness, dizziness and drowsiness. Pregnant women and children are particularly vulnerable.[6]

### SIGNS AND SYMPTOMS OF ANAEMIA[7]

**Skin**

Skin becomes pale, buccal and pharyngeal mucous membrane shows prominent paleness in conjunctivae, lips, ear lobes, palm and nail bed. Skin becomes thin and dry losing its elasticity, early greyness, thinning and loss of hair can occur. Nails become brittle and easily breakable.

**Cardiovascular system**

Anaemia can cause tachycardia and increased velocity of blood flow and cardiac murmurs are observed.

**Respiration**

Force and rate of respiration is increased sometimes it may lead to breathlessness and dyspnoea. Oxygen haemoglobin dissociation curve is shifted to right.

**Digestion**

The common symptoms of anaemia are anorexia, nausea, vomiting, abdominal discomfort and constipation.

**Metabolism**

Basal metabolic rate is increased in severe anaemia.

**Kidney**

Albuminuria is common and renal function is disturbed.

**Reproductive system**

In females, the menstrual cycle is disturbed. Menorrhagia, oligomenorrhagia or amenorrhea is accompanied with anaemia.

**Neuromuscular system**

Headache, lack of concentration, restlessness, irritability, drowsiness, dizziness or vertigo especially when standing, increased sensitivity to cold and fainting sensation are common symptoms. Muscles become weak and there is lack of energy and fatigue.

## Agents used to treat anaemia[5]

### Iron

Iron is stored in intestinal mucosal cells as ferritin. Iron deficiency results from a negative iron balance due to depletion of iron stores, acute chronic blood loss and insufficient intake during periods of accelerated growth in children, heavy menstruation and/or inadequate intake of iron in diet. Supplementation with ferrous sulphate is required to correct the deficiency.

**Adverse effect**

GI disturbance caused by local irritation are the most common adverse effects.

**Folic acid**

Folic acid is used in treating deficiency states that arise from inadequate levels of vitamin. Folate deficiency may be caused by increased demand (during pregnancy and lactation), poor absorption caused by pathology of small intestine, alcoholism or treatment with drugs that are dihydro folate reductase inhibitors. Folic acid is normally well absorbed in jejunum. Oral folic acid administration is nontoxic.

**Adverse effects**

Rare hypersensitivity reactions to parenteral injections have been reported.

**Cyanocobalamine**

Vitamin $B_{12}$ deficiency results from low dietary levels, poor absorption of the vitamin due to failure of gastric parietal cells to produce intrinsic factor or loss of receptor activity for the intestinal uptake of the vitamin. Vit.$B_{12}$ supplementation is required in the large oral doses, sublingually or once a month by the parenteral route.

**Adverse effects**

Rarely nausea, vomiting and rhinitis have been reported with the intra nasal formulation.

**Erythropoietin and Darbepoetin**

Erythropoietin is a glycoprotein normally secreted by the kidneys that regulates red blood cell proliferation and differentiation in bone marrow. Human erythropoietin is produced by rDNA technology, is effective in the treatment of anaemia caused by renal disease, anaemia associated with HIV and in some cancer patients. Darbepoetin is a long acting version of erythropoietin that has two carbohydrate chains which improves its biological activity and half-life is increased about three times that of erythropoietin and it shows decreased clearance.

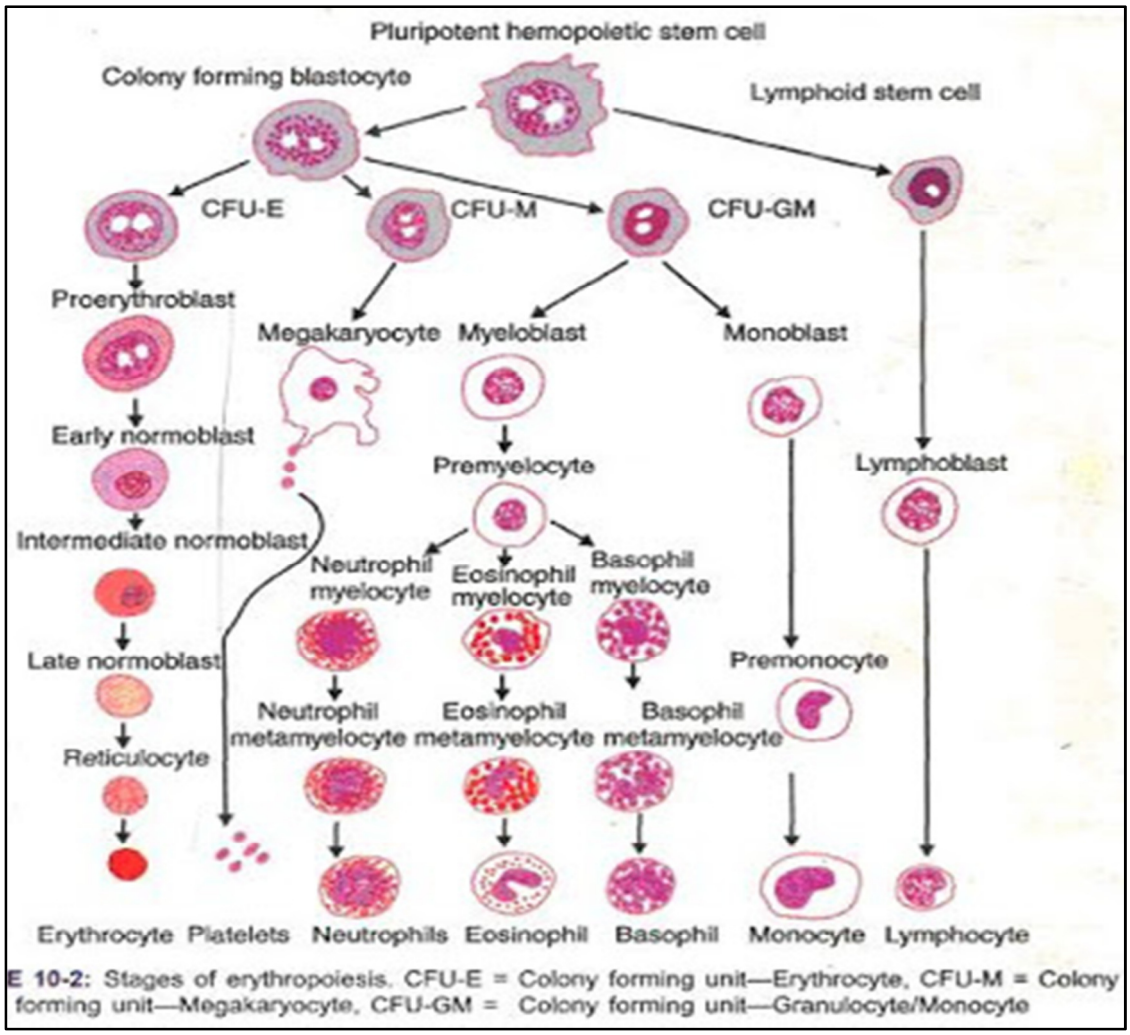
**Adverse effects**

Cause elevated blood pressure and arthralgia in some cases. When erythropoietin is used to target haemoglobin concentration more than 12g/dl serious cardiovascular events such as (thrombosis and severe hypertension), increased risk of death, shortened time of tumour progression and decreased survival has been observed.

## HAEMOPOIESIS[8]

Haemopoiesis or hematopoiesis is the process of the origin, development and maturation of all the blood cells.

**Fig.No.1 HAEMOPOISIS**




E 10-2: Stages of erythropoiesis. CFU-E = Colony forming unit—Erythrocyte, CFU-M = Colony forming unit—Megakaryocyte, CFU-GM = Colony forming unit—Granulocyte/Monocyte

## BLOOD

Blood is the connective tissue in fluid form. It is considered as the fluid of life because it carries oxygen from lungs to all parts of the body and carbon di oxide from all parts of the body to the lungs. It is also known as fluid of growth because it carries nutritive substance from the digestive system and the hormones from the endocrine gland to all the tissues. The blood is also called as fluid of health because it protects the body against the disease and gets rid of the waste products and unwanted substances by transporting them to the excretory organs like kidneys.

## BLOOD CELLS

**Three types of cells present in blood**

Red blood cells or Erythrocytes

White blood cells or Leukocytes

Platelets or Thrombocytes

## PROPERTIES OF BLOOD

**Colour**

Blood is red in colour. Arterial blood is scarlet red because it contains more oxygen and venous blood is purple red because of more carbon di oxide.

**Volume**

The average volume of blood in a normal adult is 5 litres. In new born baby it is 450 ml. It increases during growth and reaches 5 litres at the time of puberty. In females it is slightly less and is about 4.5 litres. It is about 8% of the body weight in a normal young healthy adult weighing about 70kg.

**pH**

Blood is slightly alkaline and its pH in normal conditions is 7.4.

**Specific gravity**

The specific gravity of total blood: 1.052 to 1.061

The specific gravity of blood cells: 1.092 to 1.101

The specific gravity of plasma: 1.022 to 1.026

**Viscosity**

Blood is five times more viscous than water. It is mainly due to red blood cells and plasma proteins.

**FUNCTIONS OF BLOOD**

**Nutrient function**

Nutrient substance like glucose, amino acids lipids and vitamins derived from digested food are absorbed from gastrointestinal tract and carried by blood to different parts of the body for growth and production of energy.

**Respiratory function**

Blood carries oxygen from alveoli of lungs to different tissues and carbon di oxide from tissues to alveoli.

**Excretory function**

Waste products formed from metabolic activities are remove by blood and carried out to the excretory organs like kidney, skin, liver, etc. for excretion.

**Transport of hormones and enzymes**

Endocrine hormones are released directly into blood. The blood transports these hormones to their target organs or tissues. Blood also transport enzymes.

**Regulation of water balance**

Water content of the blood is freely interchangeable with interstitial fluid. This helps in regulation of water content of the body.

**Regulation of acid-base balance**

The plasma proteins and haemoglobin act as buffers and help in regulation of acid-base balance.

**Regulation of body temperature:**

Blood is responsible for maintaining the thermo regulatory mechanism in the body since the blood has high specific heat.

**Storage function**

Water, glucose, proteins, sodium, potassium are constantly required by the tissues. And these are taken from the blood during the conditions like starvation, fluid loss, electrolyte loss, etc.

**Defensive function**

Neutrophils and monocytes in the blood engulf the bacteria by phagocytosis. Lymphocytes are involved in development of immunity. Eosinophils are responsible for detoxification, disintegration and removal of foreign proteins.

## RED BLOOD CELLS

Red blood cells are non-nucleated formed elements of blood. Red blood cells are also known as erythrocytes. The red colour of RBC is due to the presence of the colouring pigment called haemoglobin. RBC plays a vital role in transport of respiratory gasses. RBCs are larger in number compared to the other two blood cells namely white blood cells and platelets.

**Normal value**

The RBC count ranges between 4-5.5 million/cu mm of blood. In adult males it is 5 million/cu mm and in adult females it is 4.5 million/cu mm.

**Normal shape**

RBCs are disc shaped (bioconcave), the central portion is thinner and periphery is thicker. The biconcave contour of RBCs has some mechanical and functional advantages.

**Normal size**

Diameter **:** 7.2µ(6.9-7.4 µ)

Surface area : 120 sq µ

Volume : 85-90 cu µ

## HAEMOGLOBIN

Haemoglobin (Hb) is the iron containing colouring matter of RBC. It is a chromoprotein forming 95% of dry weight of RBC and 30-34% of wet weight. The function of haemoglobin is to carry the respiratory gases, oxygen and carbon di oxide. It also acts as buffer.

### Normal haemoglobin content

Average haemoglobin content in blood is 14-16g/dl. However, the value varies depending upon the age and sex of the individual.

### Structure of haemoglobin

Haemoglobin is a conjugated protein. It consists of a protein combined with an iron containing pigment. The protein part is globin and the iron containing pigment is heme. Iron is converted to stable ferric ($Fe^{+++}$) state which is attached to N-of the each pyrole ring and N-of the globin molecule.

### *Hybanthus enneaspermus*

Culturally, *Hybanthus enneaspermu*s which is called Orithalthamarai in Tamil is one of the important plants used in the treatment of anaemia. This project work deals with establishing the potency of *Hybanthus enneaspermus* as an anti-anaemic agent which will be helpful in making a healthy society.

The family Violaceae consists of 22 genera and about 900 species. *Hybanthus* is one of the genus consisting of 150 species. They have great medicinal value in Ayurveda. It contains volatile oil, anthocyanin, and flavonoids (Rutin) and carotenoid pigments[9].

*Hybanthus enneaspermus* is a small suffrutescent perennial herb, 15-30cm in height, with many diffuse or ascending branches, glaborous or more or less pubescent. The leaves are linear or lanceolate with 4.5cmand 3-8mm length and width respectively, margins are sub-sessile, entire or serrate and the stipules gland is tipped subtule. Flowers are pink to dark pink, axillary, solitary with pedicles shorter than the leaves, 6-12mm long erect, slender, bracts small above the middle of the pedicel. Sepals are 2.5mm long, lanceolate, very acute, keeled. Petals appear unequal the two upper ones are oblong, slightly longer than the sepals the 2 lateral longer, falcate the lowest much larger than the other, having an orbicular or obovate limb with a long claw which is curved behind into a short spur. Capsules are about 6mm in diameter and subglobose. Seeds are ovoid, acute, longitudinally striate, yellowish white about 1.5mm long.

The leaves are seen as tender stalks. They are demulcents and are used in decoction and as an electuary. They are employed in conjunction with some mild oil in preparing a cooling liniment for the head.

**Fig.No.2** ***Hybanthus enneaspermus***

The entire plant is used medicinally in Ayurveda, Siddha and other traditional systems of medicine for curing various ailments. It consists of steroids, triterpenes, sugars, alkaloids, phenols, flavones, catechins, tannins, anthraquinones and amino acids[10].

# Review of literature

# 2. REVIEW OF LITERATURE

In a desire to find a plant which has ability to treat and cure anaemia many plants with folklore claim for anti-anaemic activity were reviewed. Among those plants *Hybanthus enneaspermus* has an ethno medical claim in treatment of anaemia. Hence the literature review of the plant was done to find out the nature of scientific evaluations carried out on this plant.

## PHARMACOGNOSTICAL REVIEW

### Shantha T.R *et al.* (2001) Pharmacognostical studies on orilaitamarai[11]

The detailed pharmacognosy of the whole plant of Orilaitamarai (*Hybanthus enneaspermus*) included macroscopical, microscopical, histochemical and diagnostic characters of the root, stem and leaf. The physical constants, fluorescence characteristics and chromatographic studies of the whole plant are also presented.

### Raveendra Retnam K *et al.* (2007) [12]

Pharmacognostical studies of entire plant of *Hybanthus enneaspermus* was done including microscopy, macroscopy and preliminary physio-chemical investigations to identify and distinguish the plant from co-existing weeds and adulterants.

## PHYTOCHEMICAL REVIEW

### Mona R. Kukkar *et al.* (2013) Friedelane Type Triterpenoides from *Hybanthus enneaspermus* (Linn.) F. Muell. [13]

Phytoconstituents were isolated and characterized from the unsaponified petroleum ether extract of aerial parts of *Hybanthus enneaspermus* (Linn.) F. Muell., family Violaceae. Phytochemical investigation*s* showed presence of triterpenoids. The petroleum ether extract was subjected to column chromatography. Two new Friedelane type triterpenoids with molecular mass of 472 & 444 and molecular formula $C_{30}H_{48}O_4$ & $C_{30}H_{52}O_2$ respectively was isolated. The $^{13}$C-NMR spectra showed the characteristic data of the proposed triterpene structure. The compounds were proposed to be 28-hydroxy friedelane-3-one-29-oic acid and 3β-28-dihydroxy Friedelane on the basis of spectroscopic evidence, including nuclear magnetic spectroscopy as well as its IR spectrum.

**Anand T *et al.* (2013) Phytochemical Analysis of *Hybanthus enneaspermus* using UV, FTIR and GC- MS[14]**

The bioactive constituents present in ethanolic extracts of *Hybanthus enneaspermus* was characterized using UV, FTIR and GC-MS. The crude extracts were scanned in the wavelength ranging from 300-1100nm by using Perkin Elmer Spectrophotometer and the characteristic peaks were detected. The UV profile showed different peaks ranging from 300-1100nm with different absorption respectively. The FTIR spectrum confirmed the presence of phenols, alcohols, alkanes, alkyl halides, carboxylic acids, aromatics, nitro compounds and amines in ethanolic extract. GC-MS provided different peaks determining the presence of phytochemical compounds with different therapeutic activities. The major phytoconstituents were (5E,13E)-5,13-Docosadienoic acid (20.90%) and Cedran-diol, 8S, 14- (13.02) which are known to possess many biological activities.

**Thyaga Raju K *et al.* (2014) Phytochemical studies and elemental analysis[15]**

The phytochemical constituents, antioxidant properties and elemental analysis of different solvent leaf extracts of *H.enneaspermus* were investigated. The Phytochemical and qualitative analysis on different extracts of *H.enneaspermus* showed the presence of alkaloids, flavonoids, carbohydrates, steroids, tannins, glycosides and terpenoids. Radical scavenging, antioxidant activity were significant in ethanolic extract. The dried leaf powder analysis had revealed the presence of 90.6% of dry matter, 9.31% of moisture content, 35.1% of crude fibers, 12.5% of total ash, 0.3% of acid insoluble ash and 6.8% crude fat. Elemental analysis was done by ICP-OES and it revealed the presence of calcium (4.7mg), magnesium (4.2mg), zinc (3.0mg), phosphorus (3.2mg) and potassium (11.4mg) per gram of dry leaf powder.

## PHARMACOLOGICAL REVIEW

**Vetriselvan S *et al.* (2013) Anti-hyperlipidemic activity[16],**

The evaluation of anti-hyperlipidemic activity was carried out in high fat diet induced hyperlipidaemia in wistar albino rats. Significant effects were obtained from ethanolic extract of *Hybanthus enneaspermus* as evident from restoration of biochemical parameters altered by cholesterol towards the normal levels. Studies and observations on histopathology of the liver were normal in treated groups. The study was carried out using three doses and among these, the dose of 400mg/kg showed the best activity comparable with that of the standard drug atorvastatin in a dose of 1.2mg/kg.

**Nathiya S *et al.* (2013) The Anti-infertility activity[17]**

Infertility is an inability to conceive or inability to carry a pregnancy to give birth. It has been a major medical and social preoccupation. Various parameters had been evaluated such as MDA, GSH, α-tocopherol (vitamin.E), ascorbic acid, antioxidant enzyme of ALT (SGOT), AST (SGPT), urea, total cholesterol, haemoglobin, total protein, sperm count and sperm motility on Ethanolic extract of *Hybanthus enneaspermus*. The plant is used to recover the infertility in Endosulfan induced toxicity in male albino rats.

**Thamilz Mozhi M *et al.* (2013) Analgesic activity[18]**

The analgesic activity was screened using hot plate method, tail immersion and tail flick method. The Petroleum extract and alcoholic extracts were compared; the alcoholic extract had significant analgesic activity.

**Thamilz Mozhi M *et al.* (2013) Anti-allergic activity[18]**

The anti-allergic activity was screened by milk induced eosinophilia and leukocytes method. Comparing petroleum ether and alcoholic extracts, the latter showed significant anti-allergic activity. The extract possessing this activity may be due to the presence of phytochemical constituents mainly flavonoids and polar constituents.

**Patel DK *et al.* (2012) Aldose reductase inhibitory activity[19]**

Aldose reductase (AR) inhibitory activity was evaluated from Ethanolic extract and one isolated compound beta sitosterol from *Hybanthus enneaspermus*

Linn F. Muell. AR inhibitory activity against rat lens, there was a significant difference in the AR inhibitory activity, *H. enneaspermus* showed significant AR inhibitory activity compared to the isolated compound beta sitosterol, which may be due to its high phenolic and flavonoid content.

**Hemashenpagam N *et al.* (2010) Anti-microbial activity[20]**

The leaves of *Hybanthus enneaspermus* was evaluated for antimicrobial activity. The *in-vitro* antimicrobial activity was performed by agar disc diffusion method using *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Candida albicans, Pseudomonas aeroginosa* and *Klebsiella pneumonia*. The significant and broad spectrum of inhibition was exhibited by Ethanolic extract when compared with Chloroform and Petroleum ether extract comparing with the standards. Thus, the plant was observed to have antimicrobial activity.

**Manjunath Setty M *et al.* Antioxidant[6] and Free radical scavenging activity[21]**

Evaluation of antioxidant property of *Hybanthus enneaspermus* by 2, 2-Diphenyl-1-PicrylHydrazyl (DPPH) assay was carried on; DPPH assay gives a strong absorption band at 517nm in visible region. The degree of reduction in absorbance measurement is indicative of the radical scavenging power of the extract. The Ethanolic extract of *Hybanthus enneaspermus* appeared to be as potent as Butylated Hydroxy Toluene (BHT) which is used as a standard**.**

Better free radical scavenging activity was observed on alcoholic extract of *Hybanthus enneaspermus* on the concentration dependent scavenging of DPPH, nitric oxide, ABTS, superoxide and lipid peroxidation studies with the concentrations of 2µg/ml to 1024 µg/ml. This showed potent free radical scavenging activity of *Hybanthus enneaspermus*.

**Manjunath Setty M *et al.* Nephro-protective activity[21]**

Nephro protective activity was observed in cisplatin induced renal injury. Single administration of cisplatin at 5mg/kg bodyweight produced significant increase in blood urea, serum concentration and protein level followed by significant loss in body weight of the animals. Antioxidant defence system was impaired as indicated by the increase in TBARS level and decrease in GST, GSH, and SOD level in renal tissues.

Aqueous extract of *Hybanthus enneaspermus* at the dose of 500mg/kg body weight and alcoholic extract at the doses of 250mg/kg and 500mg/kg was found to normalize the raised blood urea, blood protein and serum creatinine. Further, the extracts were able to raise the cisplatin induced decreased GST, GSH, SOD and protect the kidney from lipid peroxidation damage. It was observed that $10^{th}$ day administration of 500mg/kg of alcoholic extract prior to cisplatin administration (5mg/kg, single dose) in prophylactic regimen, effectively prevented the cisplatin induced renal injury.

**Tripathy S *et al.* (2009) The Anti-arthritic activity[22]**

The alcoholic and aqueous extracts of the entire plant of *Hybanthus enneaspermus* on Freund's adjuvant induced arthritis was evaluated. The percentage of yield was found to be 12.8 and 10.6% for alcoholic and aqueous extracts respectively. Both the extracts significantly ($p<0.001$) decrease the paw thickness at the end of 30 days treatment, though both the extracts showed the same level of activity in the acute phase of inflammation. The alcoholic extract was found to be more potent than the aqueous extract in the chronic phase of inflammation. There was 57.4% - 59.4% inhibition seen with the alcoholic extract. This results support the folklore claim of the usefulness of the plant against the inflammatory conditions like arthritis.

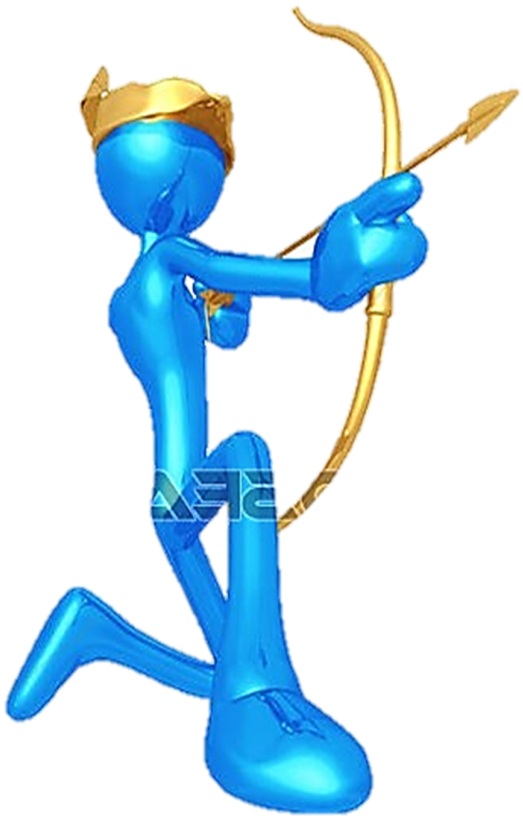
# Rationale for selection

## 3. RATIONALE FOR SELECTION

*Hybanthus enneaspermus* is a small herb under Violaceae family. It has various ethno botanical applications and medicinal claims. It is easily digested, removes kapha and pitta, urinary calculi, stangury, pain, dysentery, vomiting, burning, wandering of the mind, urethral discharge, blood troubles, asthma, epileptic fits, cures cough, gives tone to the breast, alexetric (Ayurveda).

In south west Nigeria, *Hybanthus enneaspermus* locally known as Abiwere, is used by traditional birth attendants to make delivery less laborious.

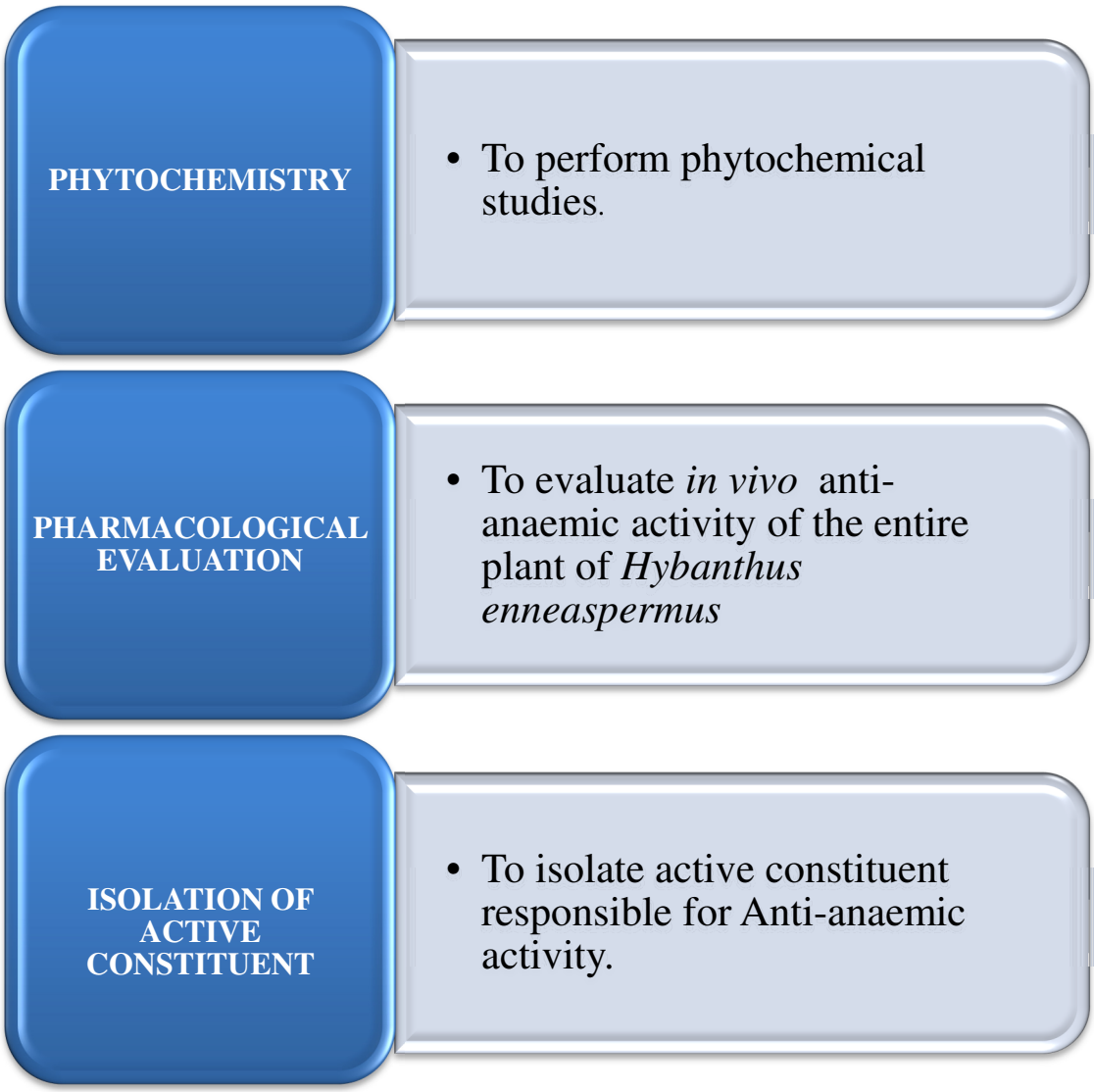
It claims to cure anaemia and improve blood quality but there is lack of scientific validation of this claim. Hence the present study was designed to prove the Anti-anaemic activity of *Hybanthus enneaspermus*.



# Aim and Objective

# 4. AIM AND OBJECTIVE

The aim of the present study is



# Plant profile

## 6. PLANT PROFILE

**Plant introduction**

Botanical name : *Hybanthus enneaspermus*

Synonym : Ionidium suffruticosum

Family : Violaceae

**VERNACULAR NAMES**[23]

**English** **:** Spade flower

**Tamil** **:** Orithalthamarai

**Sanskrit** **:** Amburuha, Atichara, Avyatha, Charati, Padma, Padmacharini, Padmavati, Padmavah, Ramya, Sthalapadmini, Supushkara.

**Bengali** **:** Munbora

**Hindi** **:** Ratanpurush

**Telugu** **:** Ratanpurusha

**Malayalam** **:** Orithalthamara

**Kannada** **:** Purusharathna

**TAXONOMY**

Botanical name : *Hybanthus enneaspermus*

Kingdom : Plantae

Phylum : Tracheophyta

Class : Magnoliopsida

Order : malphigiales

Family : Violaceae

Genus : Hybanthus

Species : *enneaspermus*

**GEOGRAPHICAL DISTRIBUTION[23]**

Found along the rocky areas and wastelands from plains to 900m in exposed places, widely found in tropical parts of India, Srilanka, Malaysia, Africa and Madagascar, South East China to tropical Australia.

**HABITAT**

Small suffrutescent herb common in arable, pasture and waste lands.

**Flower**

The flowers of *Hybanthus enneaspermus* are solitary, axillary, pink, unequal and flowering throughout the year.

**Fruit**

It consists of a subglobose capsule; seeds ribbed, 2mm. fruiting throughout the year.

**Field tips**

The conspicuous rose petal has linings of a deeper hue.

**Leaf arrangements**

It has alternate distihous

**Leaf type**

Simple

**Leaf shape**

Linear-lanceolate or elliptic

**Leaf apex**

Acute

**Leaf base**

Attenuate

**Leaf margin**

Entire

# Plan of work

# 5. PLAN OF WORK

- **Collection of plant**
- **Authentication of the plant**
- **Herbarium of the plant**
- **Preparation of extracts**

- **QUALITATIVE ANALYSIS**
  - **Preliminary phytochemical screening of powder**
  - **Preliminary phytochemical screening of extracts**
  - **Fluorescence analysis of plant powder**
  - **Fluorescence analysis of extracts**

- **CHROMATOGRAPHIC ANALYSIS**
  - **TLC of all extracts**
  - **HPTLC of all extracts**

- **QUANTITATIVE ANALYSIS**
  - **Estimation of Total alkaloids**
  - **Estimation of Total phenolic constituents**
  - **Estimation of Total steroids**
  - **Estimation of Flavonoids**
  - **Estimation of Iron content**
  - **Estimation of Heavy metal**

- **PHARMACOLOGICAL STUDIES**
  - **Evaluation of Anti-anaemic activity**

- **ISOLATION OF ACTIVE CONSTITUENT**
  - **Chemical test for isolated compound**
  - **TLC**
  - **IR spectroscopic studies**

# Methodology

# 7. METHODOLOGY

## 7.1 PHYTOCHEMICAL STUDIES

The Phytochemical investigation of a plant involves authentication and extraction of plant material; qualitative and quantitative evaluations; separation and isolation of the constituents of interest; characterisation of the isolated compound. Parallel to this may be the assessment of pharmacological activity.

### PREPARATION OF EXTRACT [24]

Extraction is the preliminary step involved in the phytochemical studies. Based on solvent's polarity metabolites are extracted and according to the solubility of the constituents in the solvent. The method of extraction is hot percolation method.

### HOT PERCOLATION METHOD

About 200g of coarsely powdered plant was extracted with solvents of increasing polarity like Hexane, Chloroform, Ethyl acetate and Ethanol at 60-70°C. Each extract was concentrated using rotary vacuum evaporator. The percentage yield, colour and consistency of all the extracts were noted and were taken up for further detailed phytochemical and pharmacological screening.

### PRELIMINARY PHYTOCHEMICAL SCREENING [25]

Qualitative analysis for various phytoconstituents in the dried powder and extracts of entire plant *Hybanthus enneaspermus* was carried out using different reagents are mentioned below.

### DETECTION OF ALKALOIDS

**Dragendroff's Test**

The powder/extract was dissolved in 5ml of distilled water, to this 5ml of 2M Hcl was added. Then 1ml of Dragendroff's reagent was added and examined for an immediate formation of an orange red precipitate.

**Mayer's Test**

The powder/extract was mixed with little amount of dilute Hcl and Mayer's reagent and examined for the formation of white precipitate.

**Wagner's Test**

The powder/extract was mixed with Wagner's reagent and examined for the formation of reddish brown precipitate.

**DETECTION OF GLYCOSIDES**

**Bontrager's Test**

The powdered plant material/extract was boiled with 1ml of sulphuric acid in a test tube for few minutes. The solution was filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of the solvent was separated and shaken with half of its volume of dilute Ammonia. Formation of a rose pink to red colour in the ammoniacal layer indicates the presence of glycosides.

**Modified Bontrager's Test**

The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% freshly prepared aqueous ferric chloride solution for 5 min and shaken with equal volume of chloroform .the lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. Formation of a rose pink to red colour in the ammoniacal layer indicates the presence of glycosides.

**Legal's Test**

The test material when treated with sodium nitroprusside in pyridine and methanolic alkali. Formation of a pink to blood red colour indicates the presence of cardiac glycosides.

**DETECTION OF STEROIDS**

**Liebermann-Buchards Test**

The powdered drug/extract was treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added from the sides of the test tube. If a brown ring is formed at the junction of two layers and upper layer turns green, it shows presence of steroids.

**Shinoda Test**

To the solution of extract few piece of magnesium turnings and drops of concentrated Hcl were added. If a pink crimson red or occasionally green to blue colour appears after few minutes, this indicates the presence of flavonoids.

**Alkaline reagent Test**

To the test solution, few drops of sodium hydroxide solution were added. If there is a formation of intense yellow colour which turns to colourless on addition of few drops of dilute acid, this indicates presence of flavonoids.

**DETECTION OF CARBOHYDRATES**

**Molisch's Test**

To the test solution, few drops of alcoholic alpha-napthol and few drops of concentrated Sulphuric acid were added through the sides of the test tube. Appearance of purple to violet colour ring at the junction indicates the presence of carbohydrates.

**Fehling's Test**

The test solution was mixed with Fehling's I and II, heated and examined for the presence of red colouration for the presence of sugar.

**DETECTION OF PHENOL**

**Ferric chloride Test**

A small quantity of powdered drug/ extract was dissolved in 2ml distilled water and a few drops of 10% aqueous ferric chloride solution was added and observed for appearance of blue or green colour.

**DETECTION OF PROTIENS**

**Biuret Test**

The sample was treated with 5-8 drops of 1% w/w copper sulphate solution and 1ml of 5% sodium hydroxide. If a violet colour is formed it indicates the presence of proteins.

**DETECTION OF TANNINS**

**Lead acetate Test**

The test solution was mixed with basic lead acetate solution and examined for formation of white precipitate.

**Ferric chloride Test**

A few drops of 5% aqueous ferric chloride solution was added to 2 ml of aqueous extract of the drug and examined for the presence of bluish black colour.

**DETECTION OF SAPONINS**

A drop of sodium bicarbonate solution was added to the sample and the mixture was shaken vigorously and left for 3minutes. Development of any honey comb like froth was examined.

**DETECTION OF GUMS AND MUCILAGE**

A small quantity of powdered drug/extract was dissolved in 5 to 10 ml of acetic anhydride by means of heat, cooled and 0.05ml of concentrated Sulphuric acid was added. Formation of bright purplish red colour indicates the presence of gums and mucilage.

**DETECTION OF FIXED OILS AND FATS**

A small quantity of extract was pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oils and fats.

## 7.2 FLUORESCENCE ANALYSIS [26, 27]

Fluorescence analysis was carried out in day light and in UV light. The powdered plant material of *Hybanthus enneaspermus* and their extracts was treated with various reagents and solvents to identify the presence of chromophores. The fluorescence was observed in day light and in short and long UV light 254nm and 365nm respectively.

## 7.3 CHROMATOGRAPHIC ANALYSIS [28, 29]

### THIN LAYER CHROMATOGRAPHY

**Principle**

It consists of a thin layer of adsorbent coated on a chromatographic plate, the mobile phase (developing solvent) flows against gravitational force by means of capillary action. The separation is mainly on the differential migration that occurs when the solvent flows along the thin layer of stationary phase. The principle involved thin layer chromatography is adsorption.

### TLC PLATE PREPARATION

The plates were prepared using Stahl TLC spreader. 40gm of silica gel G was mixed with 85ml of water to prepare homogenous suspension and poured into the spreader. 0.25mm thickness of plates was prepared, air dried until the transparency of the layer disappeared and the plates were then dried at 110°C for 30 minutes and kept in desiccators.

**Mobile phase**

Single or mixture of solvent was selected based on the phytoconstituents present in the extracts. Factors such as nature of the components, stationary phase, mobile phase, polarity influence the rate of separation of constituents. From various trials, best solvent was selected which showed good separation with maximum number of components.

### HPTLC FINGERPRINT PROFILE[30]

HPTLC is one of the advanced and versatile chromatographic technique which helps in the identification of compound and thereby authentication of purity of herbal drugs. It is very quick process. In addition to qualitative detection, HPTLC also provides semi-quantitative information on major active constituents of a drug thus enabling an assessment of drug quality.

HTLC serves as a convenient tool for finding the distribution pattern of the phytoconstituents which is unique to each plant. The finger print obtained is suitable for monitoring the identity and purity of drugs and for detecting adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and standardize the quantity of active principles present in the herbal extracts.

**INSTRUMENT CONDITIONS**

**Sample Used : All four extracts**

**Instrument : CAMAG HPTLC**

**HPTLC Applicator : CAMAG LINOMAT IV**

**HPTLC Scanner : CAMAG TLC Scanner II**

**Volume of Injection : 20 $\mu$l**

**TLC Plate : Aluminium Coated Silica Gel-Merk F254**

**Mobile Phase : Hexane:ethyl Acetate:chloroform:toluene:methanol: Formic acid(3:3:1:1:2:0.50)**

**Wave Length : 4000-800nm**

**Lamp : Tungsten Lamp**

**Chromatographic conditions**

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 10*10cm pre-activated aluminium coated silica gel-Merk F254 plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator Linomat IV with nitrogen flow CAMAG Switzerland 8mm from the bottom.

## 7.4 QUANTITATIVE ANALYSIS

**ESTIMATION OF FLAVONOIDS**[31]

**Method**: HPLC

**Extraction solvent**:

Mixture of alcohol, water and Hcl in the ratio of 50:20:8

**Mobile phase:**

A mixture of methanol, water and phosphoric acid in the ratio 100:100:1

**Preparation of Standard solutions**:

Accurately weighed quantities of quercetin RS, gallic acid, thymoquinone, gallangin are added to the separate volumetric flask, substances were dissolved in methanol, and diluted quantitatively with methanol to obtain standard solution of 1mg/ml, respectively.

**Preparation of test solution:**

About 10g of extracts were weighed accurately to a 250ml flask fitted with a reflux condenser. 78ml of extraction solvent was added and refluxed on a hot water bath for 135min and then it was allowed to cool at room temperature, the solution was decanted to a 100ml flask, 20ml of methanol was added and sonicated for 30minutes, filtered and the filtrate was made upto volume with methanol.

**Chromatographic system:**

The liquid chromatograph equipped with a 270nm detector and a 4.6mm*25cm column that consists of L1 packing. The flow rate was about 1.5ml per minute. 20µl of standard and test solutions were injected separately in to the septum, Chromatographic peak responses for standard and test were recorded and each flavonoid in the sample was estimated and tabulated.

**ESTIMATION OF PHENOLS**[32, 33, 34]

**Method:** HPLC

**Standard solution:**

5.0mg of phenol standard was dissolved in tetrahydrofuran R and diluted to10.0ml with the mobile phase.1.0ml of this solution was diluted to 20.0ml with the mobile phase.

**Test solution:**

25.0mg of the extract to be examined was dissolved in 5ml of tetrahydrofuran R and diluted to 25.0ml with the mobile phase.

**Column used:** $C_{18}$ with 200cm*4.6nm

**Stationary phase:** Octadecylsilyl silica gel for chromatography

**Mobile phase:** Glacial acetic acid: tetrahydrofuran: water (2.5:40:60)

**Flow rate:** 0.9ml/min

**Detection:** spectrophotometer at 254nm

**Injection:** 20µl

**Runtime:** 30 minutes.

## ESTIMATION OF ALKALOIDS[35, 36]

### Method

This HPLC method was applied in the validation of the ambient extraction method.

### Mobile phase:

9.93 g of monobasic potassium phosphate was dissolved in 730 ml of distilled water. 270 ml of acetonitrile was added, mixed, filtered, and de-gassed. Other necessary adjustments were made.

### Standard solution:

About 0.2mg of standard was accurately weighed and dissolved in a mixture of water and methanol in the ratio of 1 : 1.

### Test solution:

Finely powdered and 1.0 g accurately weighed sample was transferred to the flask and subjected to continuous extraction with 150 ml of methanol and extracted for 6 hours. The volume of the thimble should be at least one-half that of the volume of methanol. Cooled to room temperature and the methanol extract was transferred to a 200ml volumetric flask. Extracted flask was rinsed with methanol, the contents were transferred to the volumetric flask and diluted with methanol to volume.

### Chromatographic system:

The liquid chromatograph is equipped with a 235nm detector and a 4.6mm × 150mm column that contains packing L1.

**Flow rate:** 1.8 ml per minute.

### Procedure:

Equal volumes (about 10µl) of the Standard solution and the Test solution were separately injected into the chromatographic column. The chromatogram was recorded and the areas for the major peaks were measured. The percentage of alkaloids present in the solution was calculated.

**ESTIMATION OF STERIODS[37]**

**Standard solution:**

Accurately weighed sample to that of the reference substance specified in the individual monograph, previously dried under the specified conditions and dissolve in a suitable volume of aldehyde - free ethanol. Dilute quantitatively and stepwise with aldehyde - free ethanol to obtain a solution containing about 10µg of the steroid per ml.

**Test Solution:**

Test solutions are prepared according to specified conditions.

**Method:**

Into a glass - stoppered 50 ml conical flask, 20.0 ml of the test solution was pipetted out into two similar flasks, 20.0 ml of the standard solution and 20.0 ml of aldehyde - free ethanol(Blank) respectively was pipetted out. To each flask 2.0 ml of tetrazolium blue solution was added and mixed; to each flask 2.0 ml of the mixture of 10 volumes of tetra ethyl ammonium hydroxide solution (10%) and 90 volumes of aldehyde - free ethanol was added, mixed and allowed to stand in the dark a temperature between 25° and 35°. Exactly after 90 minutes 1.0 ml of glacial acetic acid was added to each flask and mixed. The absorbence of the solutions obtained from the test solution and the standard solution at about 525nm against the blank was measured.

## 7.5 QUANTITATIVE ESTIMATION OF IRON[38]

Estimation of iron was done by atomic spectrometry.

**Preparation of Iron standard stock solution:**

About 100 mg of iron powder was accurately weighed and transferred to a1000ml volumetric flask. It is dissolved in 25 ml of 6N hydrochloric acid and diluted with water to make up the volume.

**Preparation of Standard solution:**

2.0ml of Iron standard stock solution was transferred to separate 100ml volumetric flask. Contents of each flask was diluted with water and made up to volume and mixed to obtain solutions having known concentrations of about 2.0mg of iron per ml.

**Preparation of sample**:

1 gram of sample was transferred to a crucible and kept inside a muffle furnace maintained at about 550° for 6 to 12 hours and cooled. About 60ml of hydrochloric acid was added and boiled gently on a hot plate or steam bath for 30 minute intermittently rinsing the inner surface of the crucible with 6N hydrochloric acid. It was cooled, the contents of the crucible was quantitatively transferred to a 100ml volumetric flask. The crucible was rinsed with small portions of 6 N hydrochloric acid and the rinings were added to the flask. Diluted with water to volume, mixed and filtered, discarded the first 5 ml of the filtrate. This solution was diluted quantitatively and stepwise if necessary, with 0.125N hydrochloric acid.

**Procedure**:

The absorbance of the Standard preparations and the assay preparation was determined at the iron emission line at 248.3nm with an atomic absorption spectrophotometer (Spectrophotometry and Light-Scattering á 851ñ) equipped with an iron hollow-cathode lamp and an air-acetylene flame, using 0.125 N hydrochloric acid as the blank.

A graph was plotted using absorbance of standard Vs concentration. From this the concentration of the sample was determined.

### 7.6 QUANTITATIVE ESTIMATION OF HEAVY METALS[39]

**Instrumentation parameters:**

The quantitative analysis of heavy metals was done by the inductive coupled plasma-optical emission spectroscopy method.

**Instrument model:**

PE optima 5300 DV ICP-OES; optical system Dual view-axial or radial.

**Detector system:**

Charge coupled detector, (UV-visible detector which is maintaining at -40°C) to detect the intensity of the emission line.

**Light source:**

The Torch is positioned horizontally in the sample compartment along the central axis of the spectrometer optics. Changing from axial to radial viewing is a simple software command and is accomplished by computer control of a mirror located in the optical path. The torch assembly of this system comprises of two concentric quartz tubes.

**Standard alumina injector:** 2.0mm inner diameter

**Spray chamber:** Scott type

**Nebulizer:** cross flow gem type

**Preparation of sample by acid digestion method:**

50mg of the powder was treated with a mixture of sulphuric acid:water in the ratio of 4:1 in a kjeldahl flask and heated continuously till the solution was colourless. The sample mixture was then transferred in a 25ml volumetric flask and made upto the volume with distilled water. Blank solution was prepared as above without sample.

The standard of Arsenic, Lead, Mercury and Cadmium were prepared as per the protocol and the calibration curve was developed for each of them.

## 7.7 PHARMACOLOGICAL STUDIES

### EVALUATION OF ANTI-ANAEMIC ACTIVITY

**Experimental animals**

Healthy wistar albino rats of either sex weighing 150-220g were procured from Animal Experimental Laboratory, Madras Medical College, Chennai-03. The study was approved by the Institutional Animal Ethical Committee of Madras medical College Chennai. Which is certified by the committee for the purpose of control and supervision of experiments of animals, India (CPCSEA).**The Approval number: 18/243/CPCSEA Dated: 10-08-2015.**

The animals were kept individually in a clean and dry metallic cages and maintained in a well-ventilated animal house at a room temperature of 22±2° and for 12 hours light and 12 hours dark cycle. The animals were fed with standard pellet diet and water *ad libitum*.

**Acute toxicity studies[40, 41, 42]**

The acute toxicity studies have already been done as per OECD guide lines 423. The plant is safe upto 2000-5000mg/kg.

**Anti-anaemic activity**

Vit.$B_{12}$ syrup as standard and phenyl hydrazine to induce anaemia, all the chemicals used in the study were of analytical grade.

**Phenyl hydrazine induced anaemic model[43]**

This is one of the methods used in evaluating anti-anaemic activity. Anaemia was induced in adult wistar albino rats of either sex by oral administration of phenyl hydrazine 10mg/kg for eight days. The rats with haemoglobin concentration lower than the normal (13g/dl) were recruited for the study.

**Procedure**

The recruited animals were divided into 5 groups each containing 6 rats; they are treated with phenylhydrazine to induce anaemia from 1 to 8 days, except positive

group. The standard group receives vit.B12 as standard, group IV and V animals are treated with 200mg/kg and 400mg/kg of hexane extract of *Hybanthus enneaspermus* from 9$^{th}$ day to 60$^{th}$ day respectively. Blood is collected on initial day after induction of anaemia and 60$^{th}$ day after completion of treatment. Haematological measurements are made using suitable chemical, electrical and microscopical methods. Anti-anaemic activity of the plant was calculated by statistical methods.

**Table 1 : EXPERIMENTAL DESIGN**

| S.No | Group (N=6) | Name of the group | Treatment |
|---|---|---|---|
| 1 | I | Positive control | Receives vehicle for 60 days |
| 2 | II | Negative control | Treatment with phenyl hydrazine 10mg/kg from day 1 to day 8 orally |
| 3 | III | Standard | Treatment with vit. B12 from 9$^{th}$ day to 60$^{th}$ day orally |
| 4 | IV | Test dose 1 | Treatment with test dose 1 at the dose 200mg/kg from 9$^{th}$ day to 60$^{th}$ day orally |
| 5 | V | Test dose 2 | Treatment with test dose 2 at the dose 400mg/kg from 9$^{th}$ day to 60$^{th}$ day orally |
| Total | 30 animals | | Duration of the study is 60 days |

**Evaluation of Anti-anaemic activity-Haematological parameters** [44]

The complete blood count (CBC) was measured before the induction of anaemia, after the induction of anaemia and after drug treatment using Sysmex Automated haematology Analyser. The white blood cells, red blood cells, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration are counted using the direct current detection method with coincidence correction and flow cytometer. The haematocrit was done directly based on the red cell count and volume detection of each individual RBC. Haemoglobin analysis was conducted using Non- cyanide method.

**Statistical analysis**

The data was subjected to analysis of variance (ANOVA). P values < 0.05 was considered significant.

## 7.8 ISOLATION AND IDENTIFICATION OF RUTIN[45]

Rutin which is also known as violaquercetin has a claim to treat and cure anaemia. Since, from the above quantitative analysis hexane extract found to contain higher quantities of Rutin when compared with other extracts. Therefore Rutin is isolated from Hexane extract of *Hybanthus enneaspermus.*

**Procedure**

To 3gm of extract, 25ml of water was added and extracted with petroleum ether(50×3) then with chloroform (50×3).After extraction, the aqueous layer was collected and left to stand for 72 hours in a cold place, a yellow precipitate gets separated out of the solution. The precipitate was filtered and washed with combination of chloroform, ethyl acetate, and ethanol (50:25:25). The un-dissolved part of precipitate was dissolved in hot methanol and filtered; the filtrate was evaporated to dryness. The yellow powder of rutin was obtained**.**

**This was subjected to following evaluation:**

1. **Chemical test:**

To the isolated compound copper acetate solution was added, formation of emerald green indicates the presence of Rutin.

2. **TLC**

The isolated compound was subjected to TLC studies

Stationary phase: pre-coated aluminium sheet with silica gel G

Mobile phase: ethyl acetate: formic acid: acetic acid: water (5:3:1:1)

3. **Spectral studies**

The isolated compound was subjected to IR spectral analysis

# Results and Discussion

## 8. RESULTS AND DISCUSSION

PHYTOCHEMICAL STUDIES

**Preparation of extracts**

The shade dried entire plant of *Hybanthus enneaspermus* was extracted in soxhlet extractor successively with solvents of increasing polarity like n- Hexane, chloroform, ethyl acetate and ethanol. Each time before extracting with next solvent, the marc was dried. All the extracts were concentrated using rotary vacuum evaporator. The percentage yield was calculated for every extract in terms of dried weight of plant material. The colour and consistency of the concentrated extracts are given in table no.2

**Table 2 : Percentage yield of extracts of *Hybanthus enneaspermus***

| S.NO | EXTRACT | METHOD OF EXTRACTION | PHYSICAL NATURE | COLOUR | YIELD (%W/W) |
|---|---|---|---|---|---|
| 1. | **Hexane** | Continuous Hot percolation method using Soxhlet apparatus | Semisolid | Dark green | 2.18 |
| 2. | **Chloroform** | | Semisolid | Dark green | 2.36 |
| 3. | **Ethyl acetate** | | Semisolid | Dark green | 1.46 |
| 4. | **Ethanol** | | Semisolid | Dark brown | 1.7 |
| 5. | **Aqueous** | Cold maceration | Semisolid | Dark brown | 1.5 |

The extractive values revealed the particulars regarding solubility and polarity of the metabolites in the plant powder. Percentage yield of various extracts were as follows: n-Hexane (2.18%), chloroform (2.36%), ethyl acetate (1.4%) and ethanol (1.7%). Hexane and chloroform extract showed high extractive yield among other extracts.

## QUALITATIVE ANALYSIS

### Qualitative chemical tests

The Plant powder of *Hybanthus enneaspermus* and all the extracts were subjected to qualitative phytochemical analysis to identify the various phytoconstituents present in it, as per the standard procedures. The results are given in the table no.3

**Table 3 : Preliminary phytochemical analysis of powder and extracts of *Hybanthus enneaspermus***

| Chemical constituents | Powdered drug | Hexane Extract | Chloroform Extract | Ethyl acetate Extract | Ethanol Extract | Water |
|---|---|---|---|---|---|---|
| Steroids | + | + | + | + | + | + |
| Glycosides | - | - | - | - | - | - |
| Saponins | + | - | - | - | + | + |
| Flavonoids | + | + | + | + | + | + |
| Tannins | + | - | - | - | - | + |
| Proteins | - | - | - | - | - | - |
| Alkaloids | + | + | + | + | + | + |
| Carbohydrates | + | + | + | + | + | + |
| Terpenoids | + | - | - | - | + | + |
| Fats and oils | - | - | - | - | - | - |

+ indicates presence , - indicates absence

Qualitative preliminary phytochemical analysis was performed with different respective chemical detecting agent to detect the phytoconstituents and their presence in each extract and powder. Hexane and chloroform extracts showed the presence of steroids, flavonoids, alkaloids. Ethyl acetate extract was found to contain flavonoids, alkaloids and carbohydrates. Ethanol and aqueous extracts showed the presence of carbohydrates, flavonoids, saponins and alkaloids.

## FLUORESCENCE ANALYSIS

Fluorescence analysis for the extracts and the powdered drug were carried out with various reagents to identify the presence of chromophores. The importance of fluorescence analysis is that UV light shows the fluorescent nature of the compound whereas fluorescence cannot be observed in day light. Hence it is performed according to the standard procedures. The results are shown in Table 4 and Table 5.

**Table 4 : Fluorescence analysis of powder of *Hybanthus enneaspermus***

| Substance | Day light | Short UV | Long UV |
|---|---|---|---|
| Powder | Pale green and brown | Black | greenish brown |
| Powder+ $H_2O$ | Pale green and brown | Black | greenish brown |
| Powder +1N HCl | Pale green and brown | Black | Orange green |
| Powder +1N $HnO_3$ | Brown | Black | Orange |
| Powder + acetic acid | Pale green | Dark green | Greenish yellow |
| Powder +1N NaOH | Pale green | Black | Pale green |
| Powder +alc.NaOH | Pale green | Black | Pale green |
| Powder +1N KOH | Pale green | Black | Pale green |
| Powder +alc.KOH | Pale green | Black | Pale green |
| Powder+$H_2So_4$ | Black | Black | Black |
| Powder+$NH_4$ | Pale green | Black | Green |
| Powder+$I_2$ | Reddish brown | Black | Brown |
| Powder+$FeCl_3$ | Green | Black | Brown |
| Powder+ethanol | Pale green | Black | Greenish brown |

**Table no. 5 : Fluorescence analysis of extracts**

| Extract | Day light | Short UV | Long UV |
|---|---|---|---|
| Hexane | Dark green | Black | Dark green |
| Chloroform | Dark green | Black | Black |
| Ethyl acetate | Dark green | Black | Dark green |
| Ethanol | Dark brown | Black | Brown |

No fluorescence was observed for the powder as well as extracts indicating the absence of chromophore in the plant.

## CHROMATOGRAPHIC ANALYSIS

**Thin layer chromatography**:

All the extracts were subjected to thin layer chromatographic studies using various solvent systems. Several mobile phases were tried for the separation of maximum components by trial and error method. The solvent system selected was ethyl acetate: formic acid: acetic acid: water (5:3:1:1), ethyl acetate: n-butanol:ethanol:water (4:1:0.1:5) and ethanol: glacial acetic acid: formic acid:water(3:0.9:0.9:0.5). $R_f$ values were noted down for each selected extracts after elution by using different detecting agents such as Dragendroff's, Ninhydrin, Libberman Burchard, Con.Sulphuric acid & Ferric chloride.

The Hexane extract showed 2 spots whose $R_f$ values are 0.78 and 0.76. Chloroform extract showed one spot 0.64, whereas a spot found with Ethyl acetate extract showed one spot with $R_f$ of 0.60 and 4 spots were found in ethanol extract $R_f$ values 0.30, 0.61, 0.65, 0.73.

**Fig. no.3 TLC of Hexane extract**

**Fig. no.4 TLC OF Chloroform extract**

**Fig. no.5 TLC OF Ethyl acetate extract**

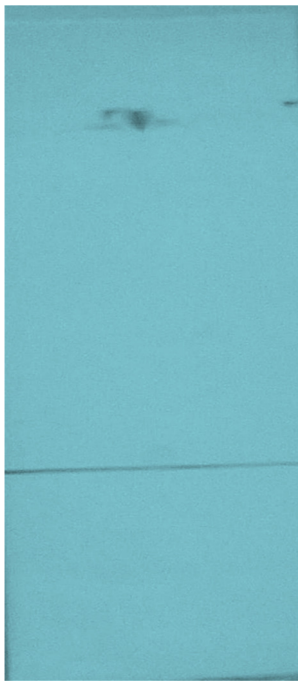
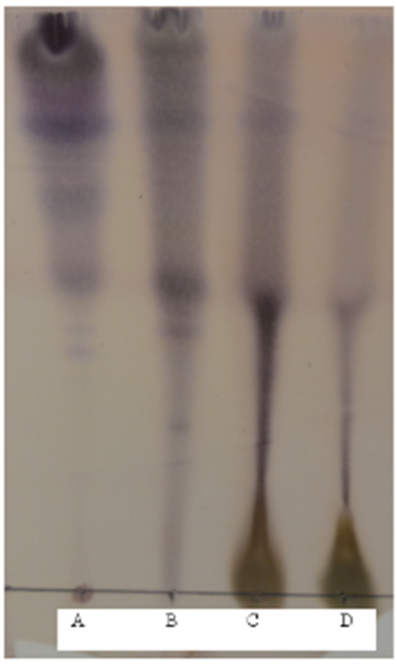


**Fig. no.6 TLC OF Ethanol extract**

**HPTLC**

The Chromatographic finger print was developed for the detection of phytoconstituents present in the each extracts and $R_f$ values were tabulated.

**Fig.No.7 HPTLC analysis of all four extracts**



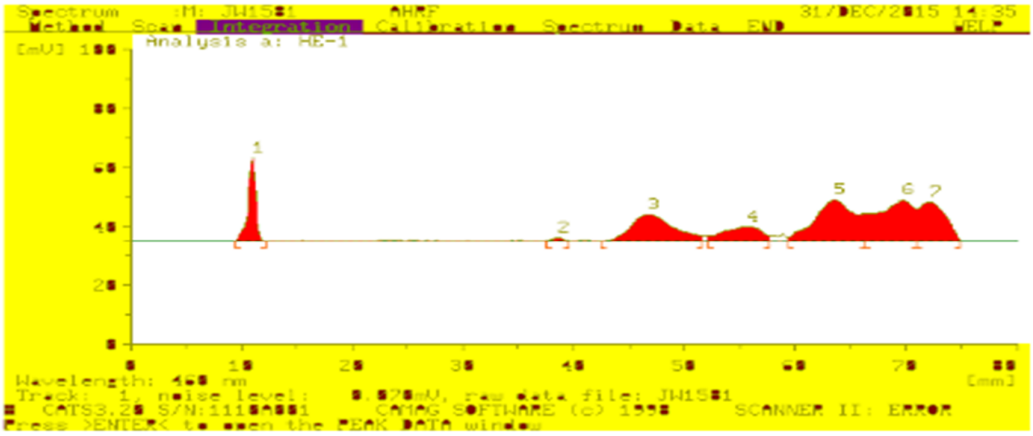

**A- Hexane extract of *Hybanthus enneaspermus***

**B- Chloroform extract of *Hybanthus enneaspermus***

**C- Ethyl acetate extract of *Hybanthus enneaspermus***

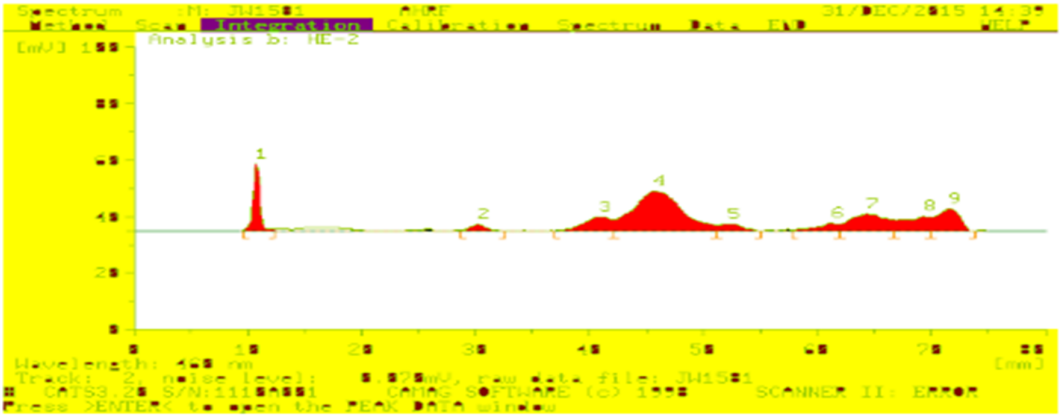
**D- Ethanol extract of *Hybanthus enneaspermus***

**Fig.no.8 HPTLC of Hexane extract of *Hybanthus enneaspermus***



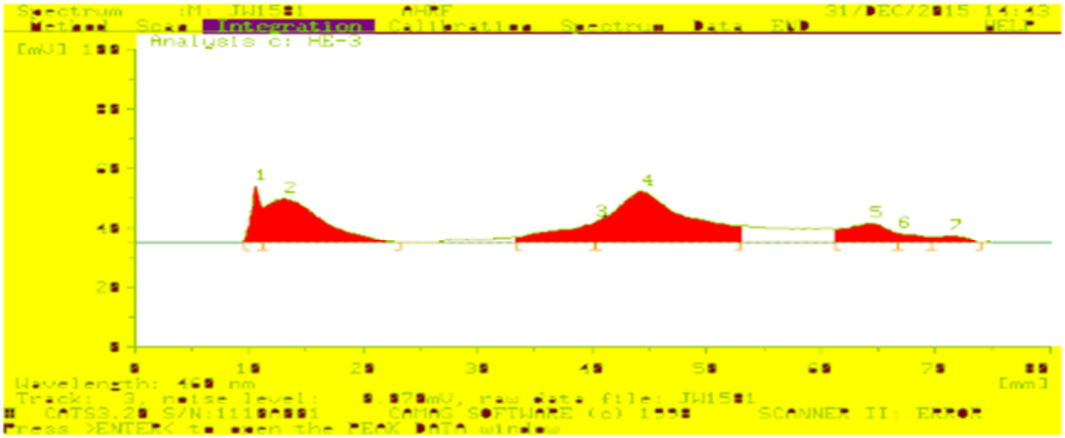

| S.No | Rf | Height | Area | Lamda Max |
|---|---|---|---|---|
| 1 | 0.11 | 28.4 | 218.2 | 699 |
| 2 | 0.38 | 1.2 | 14.9 | 599 |
| 3 | 0.46 | 9.3 | 419.1 | 530 |
| 4 | 0.55 | 5.2 | 205.6 | 598 |
| 5 | 0.63 | 14.2 | 607.7 | 601 |
| 6 | 0.69 | 14.0 | 535.6 | 428 |
| 7 | 0.72 | 13.4 | 367.9 | 427 |

**Fig.no.9 HPTLC of Chloroform extract of *Hybanthus enneaspermus***



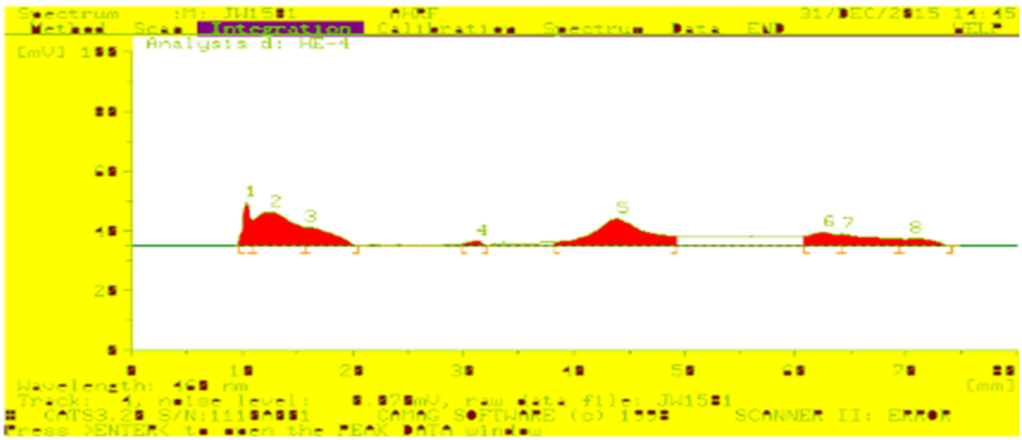

| S.No | Rf | Height | Area | Lamda Max |
|---|---|---|---|---|
| 1 | 0.11 | 23.9 | 161.4 | 712 |
| 2 | 0.30 | 2.3 | 39.4 | 570 |
| 3 | 0.41 | 5.2 | 144.2 | 526 |
| 4 | 0.45 | 14.2 | 716.7 | 430 |
| 5 | 0.52 | 2.7 | 62.0 | 419 |
| 6 | 0.61 | 2.8 | 66.5 | 429 |
| 7 | 0.64 | 6.1 | 227.6 | 428 |
| 8 | 0.69 | 5.2 | 141.6 | 428 |
| 9 | 0.71 | 8.0 | 184.8 | 422 |

**Fig.no.10 HPTLC of Ethyl acetate extract of *Hybanthus enneaspermus***



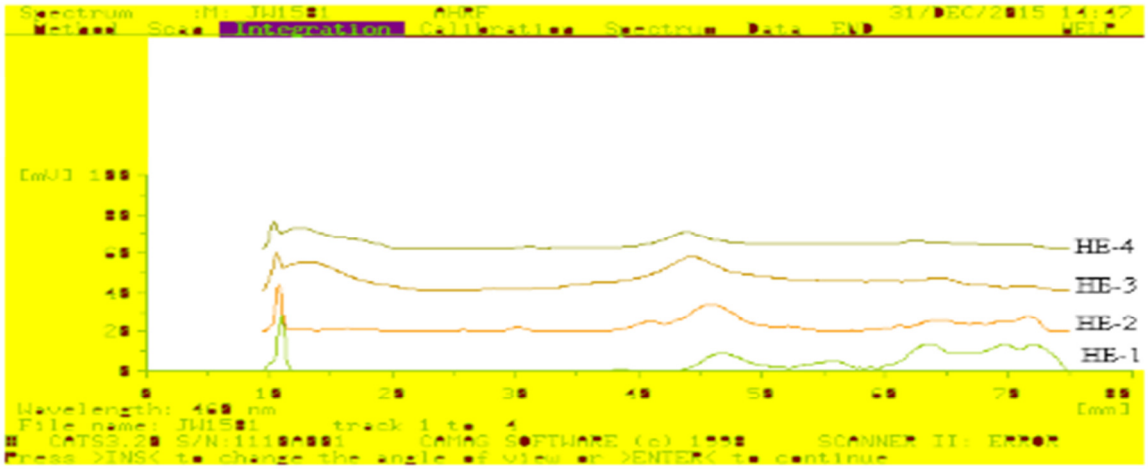

| S.No | Rf | Height | Area | Lamda Max |
|---|---|---|---|---|
| 1 | 0.10 | 19.1 | 180.4 | 452 |
| 2 | 0.13 | 14.8 | 858.3 | 400 |
| 3 | 0.40 | 7.0 | 294.5 | 436 |
| 4 | 0.44 | 17.4 | 1338.0 | 546 |
| 5 | 0.64 | 6.7 | 292.7 | 587 |
| 6 | 0.67 | 3.3 | 76.1 | 419 |
| 7 | 0.71 | 2.6 | 80.4 | 420 |

**Fig.no.11 HPTLC of Ethanol extract of *Hybanthus enneaspermus***




| S.No | Rf | Height | Area | Lamda Max |
|---|---|---|---|---|
| 1 | 0.10 | 14.8 | 113.3 | 400 |
| 2 | 0.12 | 11.5 | 448.3 | 400 |
| 3 | 0.15 | 6.3 | 179.5 | 400 |
| 4 | 0.31 | 1.6 | 25.5 | 590 |
| 5 | 0.44 | 9.2 | 520.6 | 548 |
| 6 | 0.62 | 4.6 | 132.2 | 544 |
| 7 | 0.66 | 4.0 | 153.9 | 584 |
| 8 | 0.70 | 2.5 | 84.5 | 587 |

**Fig.no.12 HPTLC of all four extracts of *Hybanthus enneaspermus***




HPTLC plate was scanned at 400-800nm with the best solvent to detect the maximum number of components and peak abundance qualitatively and quantitatively at higher resolution which is presented in fig.no.8. HPTLC fingerprint is one of the versatile tools for qualitative and quantitative analysis of active constituents of multicomponent sample and also a diagnostic method to find out the adulterants to check purity.

## QUANTITATIVE ANALYSIS

### Estimation of phytoconstituents and flavonoids

Quantitative estimation of phytoconstituents was done by HPLC equipped with 270nm detector with 4.6mm*25cm column and flow rate of 1.5ml/min. Different solvent systems were used for the separation of alkaloids, steroids, flavonoids and phenolic compounds. It is compared with the standard graph and quantified using standard formula.

**Table no. 6 : Estimation of phytoconstituents**

| Constituents | Hexane mg/ml | Chloroform mg/ml | Ethyl acetate mg/ml | Ethanol mg/ml |
|---|---|---|---|---|
| Alkaloids | 0.36 | 0.56 | 1.45 | 0.98 |
| Phenols | 0.37 | 1.67 | 3.06 | 5.38 |
| Steroids | 0.43 | 0.39 | 0.39 | 0.44 |
| **Flavonoids** | | | | |
| Quercetin | 0.101 | 0.021 | 0.046 | 0.145 |
| Rutin | 0.618 | 0.365 | 0.107 | 0.355 |
| Gallic acid | 0.201 | 0.314 | 0.462 | 0.355 |
| Thymoquine | Nil | Nil | 0.111 | 0.031 |
| Gallangin | Nil | Nil | 0.144 | 0.166 |

**Fig no.13 HPLC of Hexane extract of *Hybanthus enneaspermus***

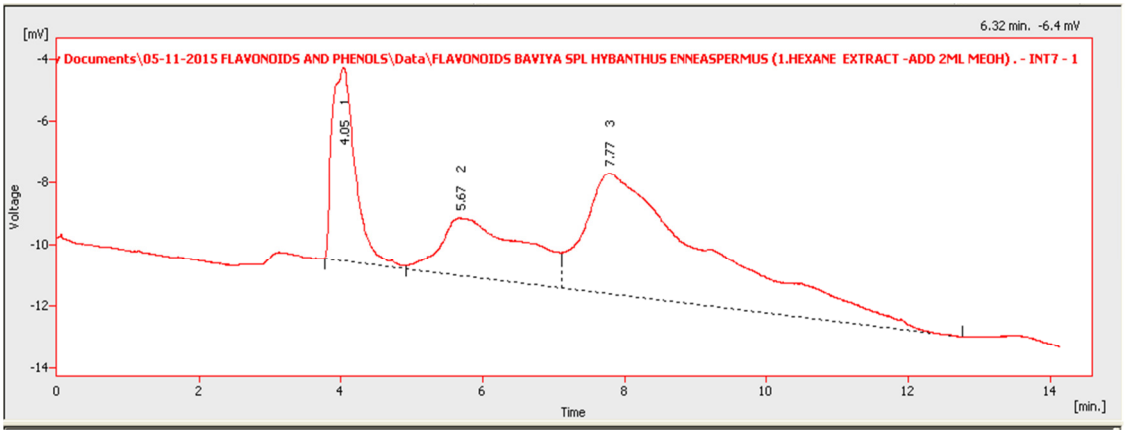


**Fig no.14 HPLC of Chloroform extract of *Hybanthus enneaspermus***

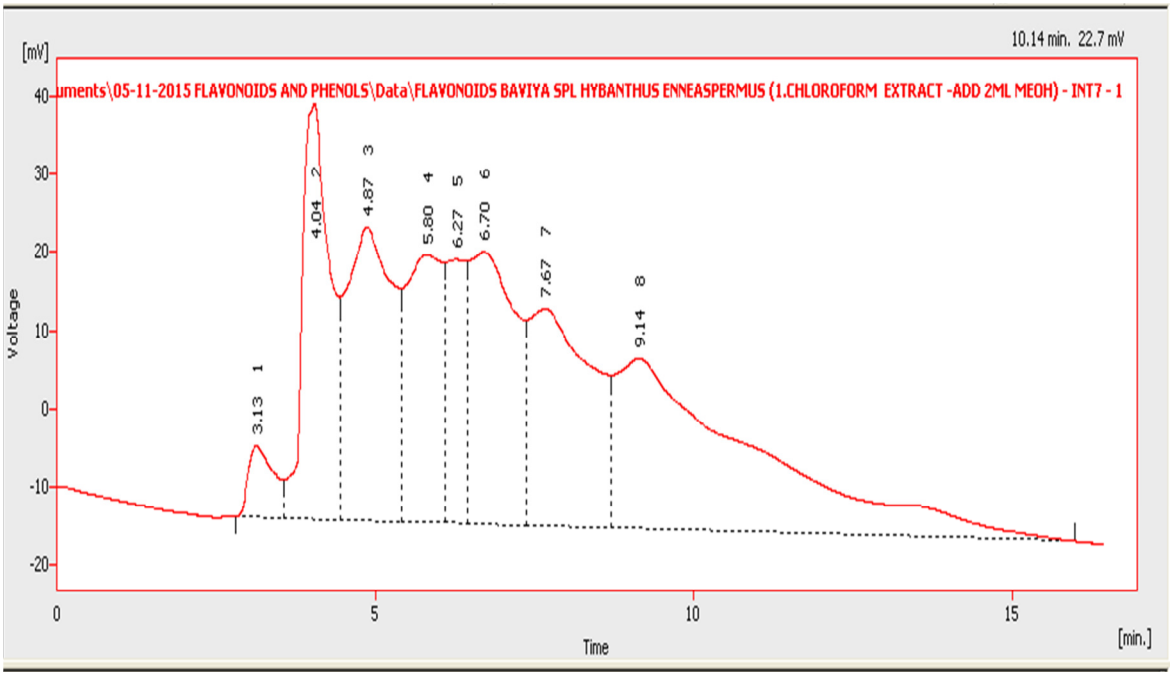



**Fig no.15 HPLC of Ethyl acetate extract of *Hybanthus enneaspermus***

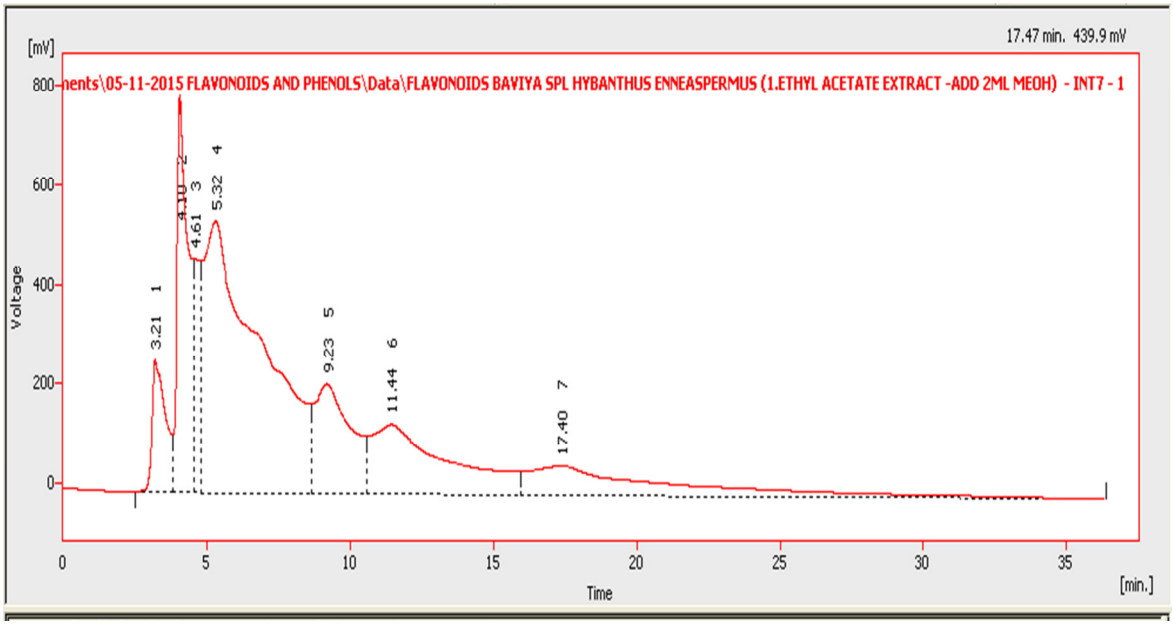
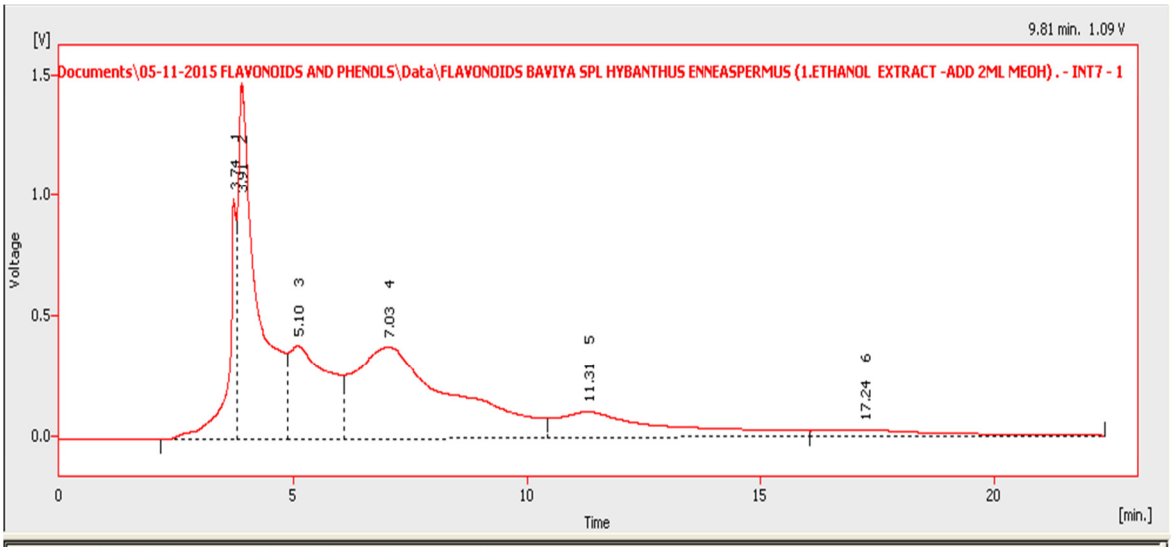


**Fig no.16 HPLC of Ethanol extract of *Hybanthus enneaspermus***




The ethyl acetate was found to be rich in alkaloids and phenols. Rutin was present in good quantities in hexane extract followed by chloroform and ethanol extract. The ethanolic extract was rich in phenolic compounds and rutin. Also, the chloroform extract had good quantities of phenol and Rutin and the hexane extract showed the highest concentration of Rutin.

**Quantitative estimation of iron**

Iron is important for the formation of haemoglobin and myoglobin. Iron is also necessary for the formation of other substance like cytochrome, cytochrome oxidase, peroxidase and catalase. The total quantity of iron in the body is 4 grams. *Hybanthus enneaspermus* is a nonheme iron dietary source that can contribute to formation of haemoglobin.

**Table 7 : Estimation of iron in various extracts**

| Compound | Hexane | Chloroform | Ethyl acetate | Ethanol |
|---|---|---|---|---|
| Iron mg/gm | 0.031 | 0.02 | 0.155 | 0.098 |

The Ethyl acetate extract showed the highest concentration of iron followed by ethanolic extract.

**Estimation of heavy metals**

Heavy metals in the sample was digested by wet digestion or dry digestion or high pressure microwave digestion and the amount of heavy metals were determined, i.e. arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) by using graphite furnace atomic absorption spectrophotometer (GF-AAS) and flow injection analysis system-atomic absorption spectrophotometer (FIAS-AAS) and the values were compared with the WHO standards.

**Table No.8 : Estimation of heavy metals**

| Element | Hexane | Chloroform | Ethyl acetate | Ethanol | Specification as per WHO Guidelines |
|---|---|---|---|---|---|
| Mercury | Nil | Nil | Nil | Nil | Not more than 0.5ppm |
| Arsenic | Nil | Nil | Nil | Nil | Not more than 5.0ppm |
| Lead | 0.04 | 0.05 | 0.08 | 0.22 | Not more than 10ppm |
| Cadmium | Nil | Nil | Nil | Nil | Not more than 0.3ppm |

The estimation of heavy metals in the sample revealed heavy metals are within the prescribed limits. It is safe and does not cause any harm on consumption.

## PHARMACOLOGICAL ACTIVITY

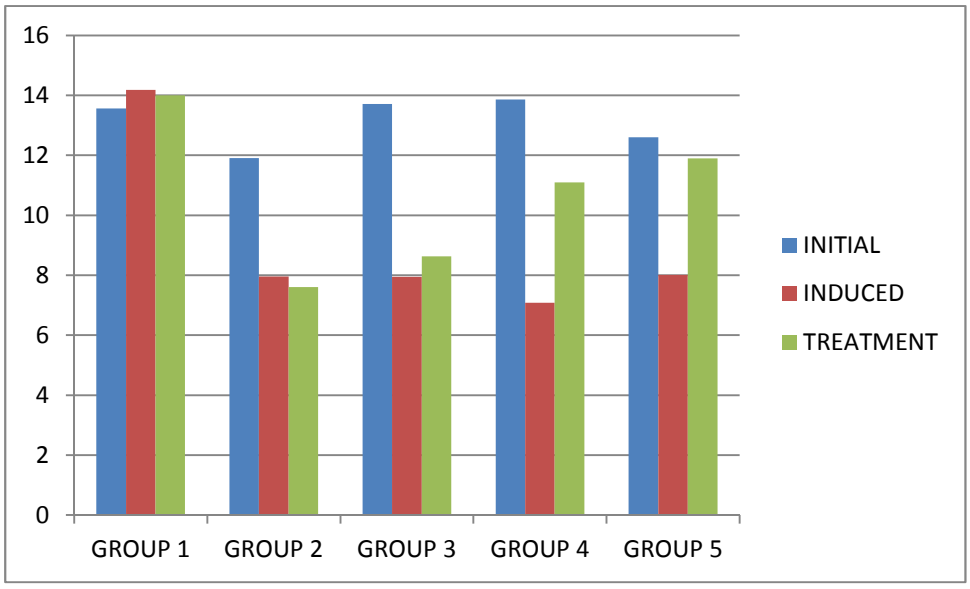
EVALUATION OF ANTI-ANAEMIC ACTIVITY

Effect of Hexane extract (200mg/kg and 400mg/kg) on the haematological parameters compared with that of the standard in following tables and graphs.

**Table 9 : Effect of n-Hexane extract on WBC count**

| SI.NO | PARAMETERS | GROUP 1 | GROUP 2 | GROUP 3 | GROUP 4 | GROUP 5 |
|---|---|---|---|---|---|---|
| 1 | INITIAL | 13.566± 3.92** | 11.916± 3.60* | 13.716± 3.77* | 13.866± 2.34*** | 12.6± 2.43*** |
| 2 | INDUCED | 14.183± 3.25** | 7.966± 1.11* | 7.95± 1.75* | 7.0833± 0.40*** | 8.016± 0.69*** |
| 3 | TREATMENT | 14±3.57** | 7.6± 0.85* | 8.633± 1.72* | 11.1± 0.85*** | 11.983± 1.34*** |

**Fig. no. 17 Graphical representations of changes in WBC COUNT**

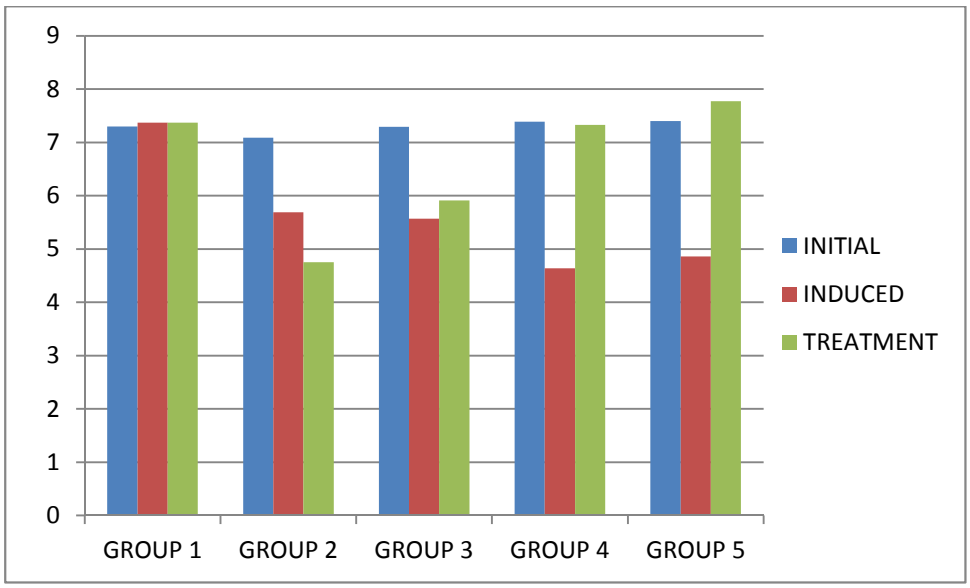



*P< 0.005, **p< 0.01, ***p< 0.0001 as compared to positive control and negative control .The data was analysed using one way Analysis of Variance (ANOVA).

**TABLE 10 : Effect of n-Hexane extract on RBC count**

| SI. NO | PARAMETERS | GROUP 1 | GROUP 2 | GROUP 3 | GROUP 4 | GROUP 5 |
|---|---|---|---|---|---|---|
| 1 | INITIAL | 7.3± 0.45** | 7.09± 0.65* | 7.29± 0.57*** | 7.39± 0.53*** | 7.4± 0.44*** |
| 2 | INDUCED | 7.37± 0.43** | 5.69± 0.69* | 5.57± 0.47*** | 4.64± 0.55*** | 4.86± 0.74*** |
| 3 | TREATMENT | 7.37± 0.27** | 4.75± 0.59* | 5.91± 0.58*** | 7.33± 0.63*** | 7.77± 0.81*** |

**Fig. no. 18 Graphical representations of changes in RBC COUNT**

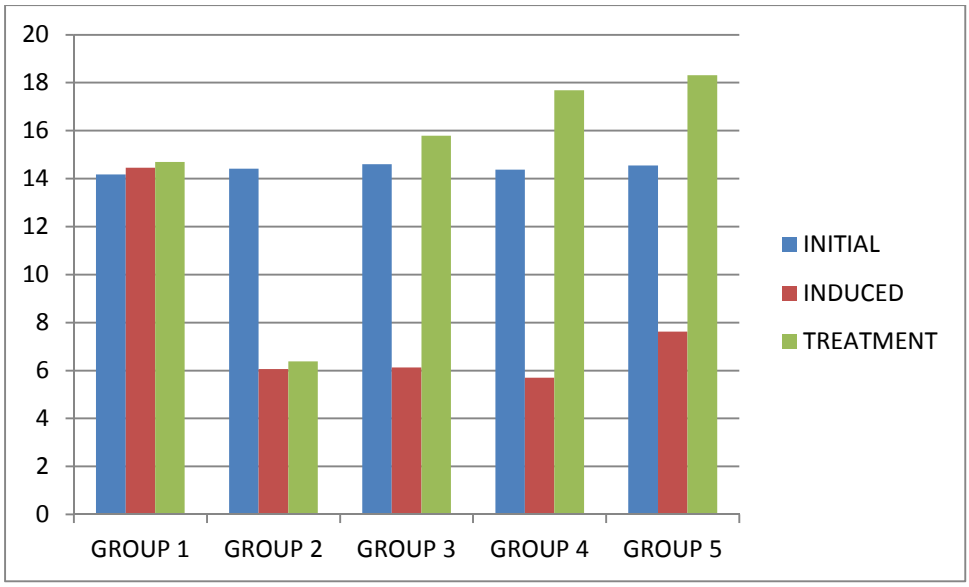



*P< 0.005, **p< 0.01, ***p< 0.0001 as compared to positive control and negative control .The data was analysed using one way Analysis of Variance (ANOVA).

**TABLE 11 : Effect of n-Hexane extract on Haemoglobin**

| SI. NO | PARAMETERS | GROUP 1 | GROUP 2 | GROUP 3 | GROUP 4 | GROUP 5 |
|---|---|---|---|---|---|---|
| 1 | INITIAL | 14.18± 0.53** | 14.41± 0.74* | 14.6± 0.36*** | 14.38± 0.53*** | 14.55± 0.75*** |
| 2 | INDUCED | 14.45± 0.57** | 6.0566± 0.49* | 6.128± 0.26*** | 5.703± 0.46*** | 7.618± 0.55*** |
| 3 | TREATMENT | 14.7± 0.61** | 6.38± 0.36* | 15.79± 0.60*** | 17.69± 1.50*** | 18.31± 1.54*** |

**Fig. no. 19 Graphical representation of changes in Haemoglobin**

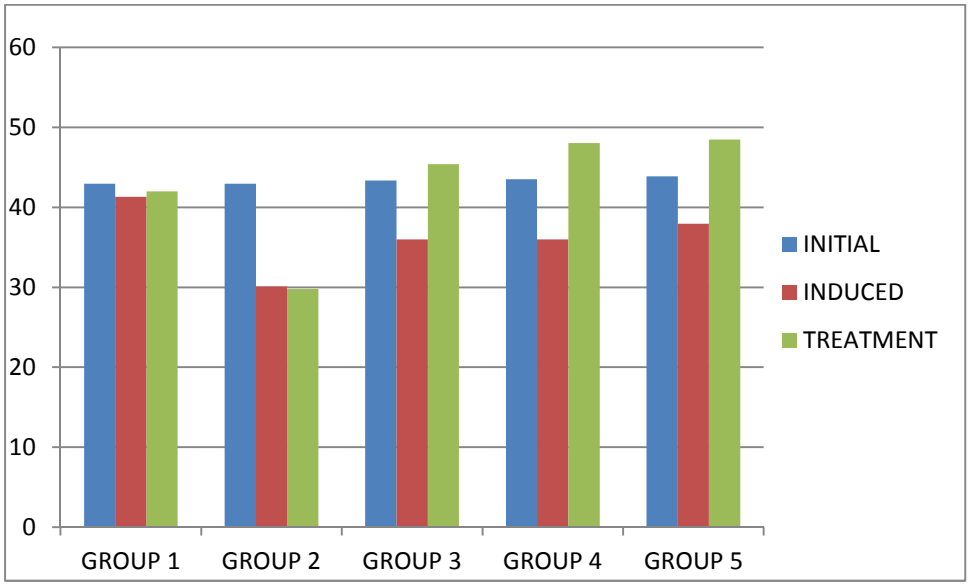



*P< 0.005, **p< 0.01, ***p< 0.0001 as compared to positive control and negative control .The data was analysed using one way Analysis of Variance (ANOVA).

**TABLE 12 : Effect of n-Hexane extract on Haematocrit**

| SI. NO | PARAMETERS | GROUP 1 | GROUP 2 | GROUP 3 | GROUP 4 | GROUP 5 |
|---|---|---|---|---|---|---|
| 1 | INITIAL | 42.95± 2.25** | 42.96± 1.65* | 43.366± 5.50*** | 43.53± 1.96*** | 43.87± 4.84*** |
| 2 | INDUCED | 41.31± 4.48** | 30.1± 1.51* | 36± 3.44*** | 35.97± 2.86*** | 37.93± 4.34*** |
| 3 | TREATMENT | 41.99± 4.73** | 29.8± 1.72* | 45.39± 4.16*** | 48.031± 5.48*** | 48.49± 2.48*** |

**Fig. no. 20 Graphical representations of changes in Haematocrit**

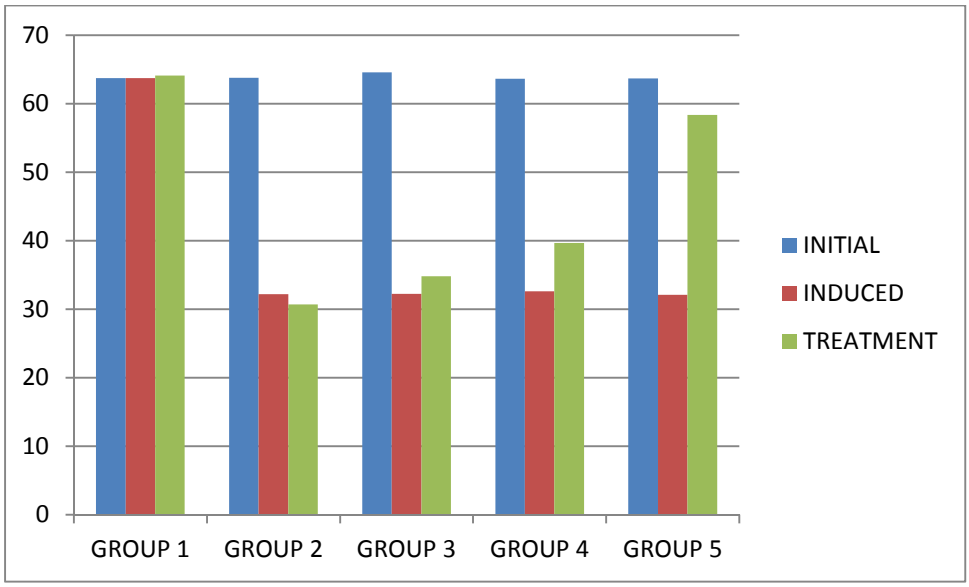



*P< 0.005, **p< 0.01, ***p< 0.0001 as compared to positive control and negative control .The data was analysed using one way Analysis of Variance (ANOVA).

**TABLE 13 : Effect of n-Hexane extract on MCV**

| SI.NO | PARAMETERS | GROUP 1 | GROUP 2 | GROUP 3 | GROUP 4 | GROUP 5 |
|---|---|---|---|---|---|---|
| 1 | INITIAL | 63.7± 1.90** | 63.77± 1.86* | 64.56± 1.53*** | 63.63± 1.97*** | 63.68± 1.83*** |
| 2 | INDUCED | 63.73± 1.089** | 32.25± 3.62* | 32.23± 2.92*** | 32.63± 2.71*** | 32.08± 4.03*** |
| 3 | TREATMENT | 64.1± 1.04** | 30.71± 6.097* | 34.83± 3.34*** | 39.65± 4.65*** | 58.33± 6.45*** |

**Fig. no. 21 Graphical representations of changes in MCV**

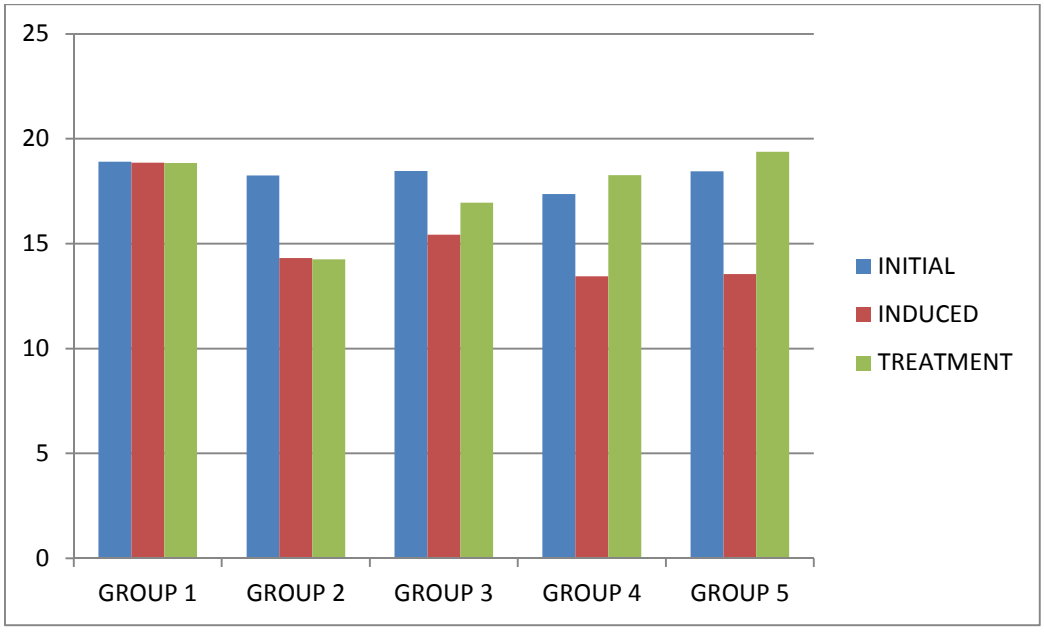



*P< 0.005, **p< 0.01, ***p< 0.0001 as compared to positive control and negative control .The data was analysed using one way Analysis of Variance (ANOVA).

**TABLE 14 : Effect of n-Hexane extract on MCH**

| SI.NO | PARAMETERS | GROUP 1 | GROUP 2 | GROUP 3 | GROUP 4 | GROUP 5 |
|---|---|---|---|---|---|---|
| 1 | INITIAL | 18.9± 1.33** | 18.25± 0.74* | 18.46± 1.69*** | 17.371± 1.29*** | 18.45± 1.33*** |
| 2 | INDUCED | 18.86± 1.16** | 14.31± 1.39* | 15.43± 0.81*** | 13.45± 0.87*** | 13.55± 0.85*** |
| 3 | TREATMENT | 18.85± 1.06** | 14.25± 1.07* | 16.95± 0.83*** | 18.26± 1.675*** | 19.38± 1.40*** |

**Fig. no. 22 Graphical representations of changes in MCH**

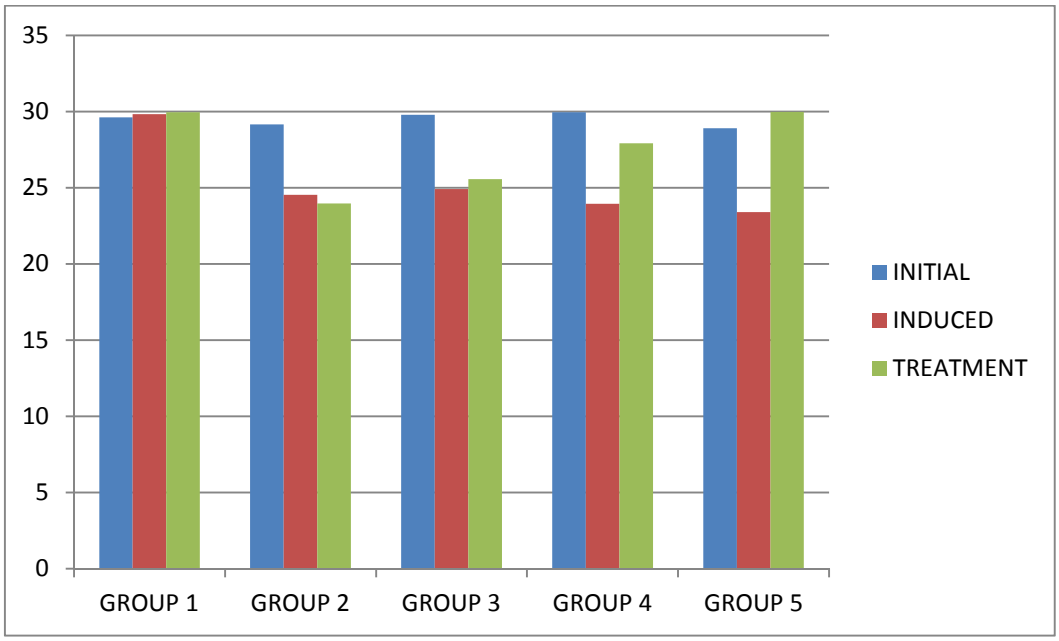



*P< 0.005, **p< 0.01, ***p< 0.0001 as compared to positive control and negative control .The data was analysed using one way Analysis of Variance (ANOVA).

**TABLE 15 : Effect of n-Hexane extract on MCHC**

| SI.NO | PARAMETERS | GROUP 1 | GROUP 2 | GROUP 3 | GROUP 4 | GROUP 5 |
|---|---|---|---|---|---|---|
| 1 | INITIAL | 29.61± 1.02** | 29.16± 0.63* | 29.78± 1.26*** | 29.96± 1.56*** | 28.9± 1.8*** |
| 2 | INDUCED | 29.83± 1.021** | 24.53± 1.69* | 24.91± 2.221*** | 23.95± 1.35*** | 23.4± 1.42*** |
| 3 | TREATMENT | 29.95± 1.005** | 23.96± 1.42* | 25.56± 2.26*** | 27.91± 2.07*** | 29.97± 4.14*** |

**Fig. no. 23 Graphical representations of changes in MCHC**




*P< 0.005, **p< 0.01, ***p< 0.0001 as compared to positive control and negative control .The data was analysed using one way Analysis of Variance (ANOVA).

From the above pictorial representations, it can be seen that in the negative control group of animals, there was a decrease in all blood parameters compared to positive control group. In the standard treated group these levels are slightly returned. There was an improvement in these values in animals treated with 200mg/kg of hexane extract and the group treated with 400mg/kg of hexane extract showed much

better improvement in all the parameters of blood. Hence *Hybanthus enneaspermus* has Anti-Anaemic activity.

**ISOLATION AND IDENTIFICATION OF RUTIN**

Rutin is also called as Rutoside, quercetin-3-o-rutinoside and sophorin. In human, it attaches to the ion $Fe^{2+}$ preventing it from binding to hydrogen peroxide which would otherwise create a highly reactive free radical that may damage cells. Rutin is an antioxidant and has vitamin P action hence used in capillary fragilities.

CHEMICAL TEST:

The isolated compound gave emerald green on treating with copper acetate.

TLC:

Stationary phase: pre-coated alumina plates

Mobile phase: Ethyl acetate: formic acid: acetic acid: water (5:3:1:1)

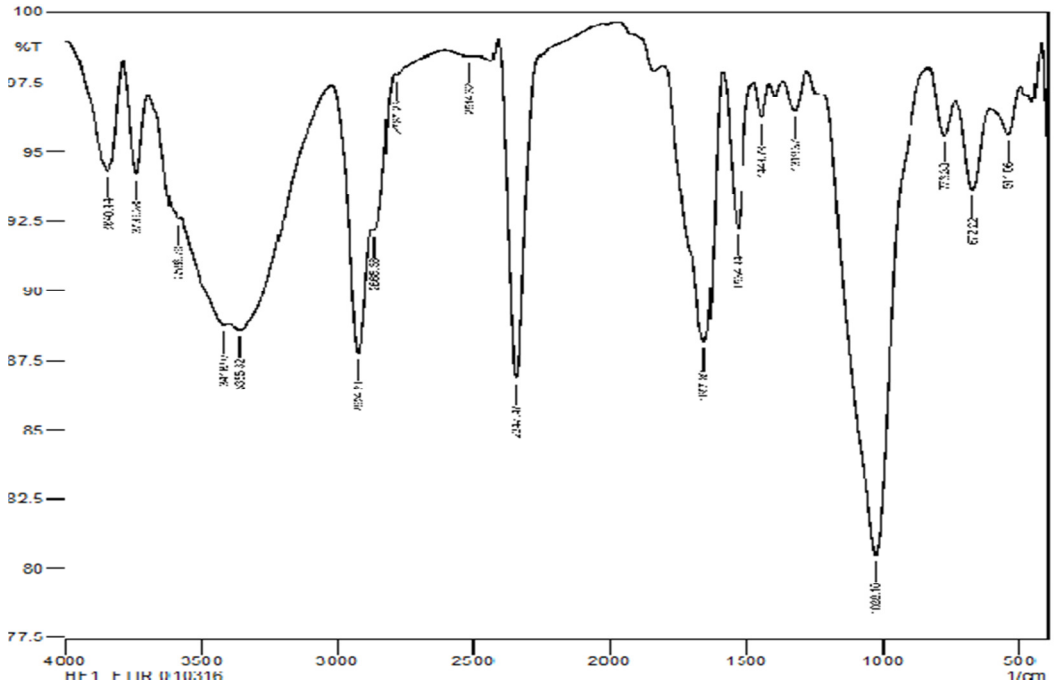
$R_f$ value:0.76

**Fig.no. 24 TLC of isolated Rutin**

The $R_f$ value of the isolated compound matches with the standard Rutin. Hence the compound isolated from hexane extract of *Hybanthus enneaspermus* was Rutin.

IR SPECTROSCOPY:

**Fig.no.25 IR spectrum of isolated Rutin**




| | Peak | Intensity | Corr. Inte | Base (H) | Base (L) | Area | Corr. Are |
|---|---|---|---|---|---|---|---|
| 1 | 541.08 | 95.622 | 1.358 | 597.96 | 497.66 | 1.854 | 0.295 |
| 2 | 672.22 | 93.602 | 3.071 | 734.91 | 608.57 | 2.696 | 0.838 |
| 3 | 776.38 | 95.50 | 1.763 | 831.36 | 735.87 | 1.473 | 0.307 |
| 4 | 1028.1 | 80.463 | 17.116 | 1224.85 | 832.32 | 17.97 | 13.791 |
| 5 | 1319.37 | 96.455 | 1.305 | 1364.7 | 1277.9 | 1.126 | 0.27 |
| 6 | 1443.78 | 96.209 | 1.305 | 1469.82 | 1416.78 | 0.741 | 0.162 |
| 7 | 1534.44 | 92.23 | 5.405 | 1591.34 | 1487.18 | 2.263 | 1.19 |
| 8 | 1657.89 | 88.142 | 9.827 | 1809.31 | 1592.31 | 6.933 | 5.039 |
| 9 | 2347.47 | 86.86 | 12.242 | 2412.00 | 2024.38 | 5.395 | 4.21 |
| 10 | 2514.32 | 98.452 | 0.034 | 2606.91 | 2493.1 | 0.74 | 0.012 |
| 11 | 2787.26 | 97.777 | 0.053 | 2794.07 | 2607.87 | 1.405 | 0.003 |
| 12 | 2865.38 | 92.16 | 0.713 | 2875.02 | 2795.94 | 1.921 | 0.196 |
| 13 | 2924.21 | 87.773 | 6.131 | 3022.58 | 2875.99 | 5.087 | 1.678 |
| 14 | 3355.32 | 88.572 | 1.274 | 3402.58 | 3023.55 | 13.118 | 1.365 |
| 15 | 3418.97 | 88.731 | 0.38 | 3578.11 | 3403.51 | 7.795 | 0.371 |
| 16 | 3586.79 | 92.581 | 0.132 | 3600.29 | 3579.07 | 0.702 | 0.007 |
| 17 | 3736.28 | 94.181 | 3.438 | 3781.5 | 3693.81 | 1.63 | 0.684 |
| 18 | 3840.44 | 94.298 | 4.158 | 3993.78 | 3785.46 | 3.006 | 1.742 |

**TABLE 16 : IR SPECTROSCOPY**

| ISOLATED RUTIN cm-1 | REFERENCE RUTIN $cm^{-1}$ | STRETCHING AND BENDING |
|---|---|---|
| 3418.97 | 3423 | OH stretch |
| 2924.21 | 2909 | C-H stretch |
| 1443.78 | 1457 | C-H bend |
| 1657.89 | 1656 | C=O |
| 1534.44 | 1505 | C=C |

The hexane extract was used for isolation of Rutin. The compound was isolated and identified by chemical test, TLC, and IR spectrum analysis. The results observed matches with previously isolated Rutin.

# Summary and conclusion

# 9. SUMMARY AND CONCLUSION

Though there are several allopathic medicines available, people still rely on nature which has blessed human kind with abundant medicinal plants for prevention and cure of disease.

The prevalence of anaemia is increasing worldwide. Though medicines are available for the treatment of anaemia in allopathic system of medicine, its compliance is an issue due to side effects.

Therefore this study is an attempt to evaluate the folklore claims of *Hybanthus enneaspermus* for the treatment of anaemia.

The review on literature shows that only few works has been done on this plant and still no anti-anaemic activity had been reported so far.

**PHYTOCHEMICAL STUDIES**

The phytochemical studies on the entire plant of *Hybanthus enneaspermus* was carried out to bring its importance as a valuable medicinal plant.

Successive solvent extraction was carried out in the order of increasing polarity n-Hexane, chloroform, ethyl acetate and ethanol. The n-Hexane and chloroform extract showed maximum yield percentage 2.18%, 2.36% w/w respectively.

The plant powder and extracts were subjected to preliminary phytochemical analysis. n-Hexane and chloroform extracts showed the presence of steroids, flavonoids, alkaloids. Ethyl acetate extract was found to contain flavonoids, alkaloids and carbohydrates. Ethanol and aqueous extracts showed the presence of carbohydrates, flavonoids, saponins and alkaloids.

Fluorescence analysis was carried out to detect the presence of chromophore present in the powder and extracts. No fluorescence was observed for powder as well as extracts.

Thin layer chromatography was performed for all the extracts of *Hybanthus enneaspermus*. Hexane extract showed two spots, chloroform and ethyl acetate extracts came out with single spot and ethanol extract had four spots.

High performance thin layer chromatography (HPTLC) finger printing was performed with all the extracts of *Hybanthus enneaspermus*. There were 7 peaks, n-Hexane and chloroform showed sharp peaks.

Quantification of phytoconstituents like estimation of total alkaloid, estimation of total phenols, estimation of steroids and individual estimation of flavonoids by HPLC showed high amount of alkaloid in ethyl acetate extract, phenols and steroids in ethanolic extract and flavonoid rutin in hexane extract.

Quantification of iron was done by atomic absorption spectroscopy revealed ethyl acetate extract had high amount of iron followed by ethanolic extract.

Estimation of heavy metals was done by inductive coupled plasma-optical emission spectroscopy method showed all the heavy metals are within limits as prescribed by WHO standards.

**PHARMACOLOGICAL STUDIES**

The *in-vivo* Anti-anaemic activity was carried out by phenyl hydrazine induced anaemic model using hexane extract. Two doses of the n-Hexane extract i.e 200mg/kg and 400mg/kg orally were used for anti-anaemic evaluation. The studies indicates that n-Hexane at the dose of 400mg/kg has better anti-anaemic activity. The haematological studies also showed increase in RBC and haemoglobin content and the overall improvement in blood quality. These changes are markedly good at higher test dose.

Hence, from these studies it is concluded that the entire plant *Hybanthus enneaspermus* possess anti-anaemic activity.

**ISOLATION AND IDENTIFICATION**

The active compound responsible for the anti-anaemic activity was isolated and been identified by chemical test, TLC and IR spectroscopy.

It shows that Rutin has Anti-anaemic activity and iron present in *Hybanthus enneaspermus* can contribute to formation of haemoglobin. Hence these data will be useful for proceeding human studies and establishing *Hybanthus enneaspermus* as an Anti-anaemic agent.

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# Annexures

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**Date: 14-07-2015**

## Certificate of Botanical Authentication

Certified that the following plant drug taken for Dissertation project work by **Ms. S.C.Baviya** M.Pharm. final year. Department of Pharmacognosy. Madras Medical College. Chennai is identified and authenticated through Visual inspection. Experience. Education & Training. Organoleptic characters, Morphology and Taxonomy methods as

***Hybanthus enneaspermus*** (L.F). MUELL. (Violaceae). Entire plant

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Reference:

D.J.Mabberley, The Plant-Book. A portable dictionary of the vascular plants. Second edition. P.351

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This is to certify that Ms. **S. C. BAVIYA,** M.Pharm II year, Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai – 600003 had submitted her protocol (Part B Application) 18|243|CPCSEA for the dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai – 600003.

**TITLE: PHYTOCHEMICAL SCREENING AND EVALUATION OF ANTI-ANAEMIC ACTIVITY OF ENTIRE PLANT OF *Hybanthus enneaspermus* Linn. (F) Muell.**

The Animal Ethical Clearance Committee experts screened her proposal No: 18|243|CPCSEA and have given clearance in the meeting held on 10|08|2015 at Dean's Chamber in Madras Medical College, Chennai – 600003. Her study involves only Wistar albino rats.

**Signature**

4/3/2016.

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